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(71) Applicant: ZYMOGENETICS, INC. [US/US]; 1201 Eastlake Avenue East, Seattle, WA 98102 (US).

(72) Inventor: CONKLIN, Darrell, C.; 117 East Louisa Street #421, Seattle, WA 98102 (US).

(74) Agent: PARKER, Gary, E.; ZymoGenetics, Inc., 1201 Eastlake Avenue East, Seattle, WA 98102 (US).

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(54) Title: HELICAL CYTOKINE ZALPHA48

(57) Abstract: Novel cytokine polypeptides, materials and methods for making them, and method of use are disclosed. The polypeptides comprise at least nine contiguous amino acid residues of SEQ ID NO:2, and may be prepared as polypeptide fusions comprise heterologous sequences, such as affinity tags. The polypeptides and polynucleotides encoding them may be used within a variety of therapeutic, diagnostic, and research applications.

Description

HELICAL CYTOKINE ZALPHA48

BACKGROUND OF THE INVENTION

Cytokines are polypeptide hormones that are produced by a cell and affect the growth or metabolism of that cell or another cell. In multicellular animals, cytokines control cell growth, migration, differentiation, and maturation. Cytokines play a role in both normal development and pathogenesis, including the development of solid tumors.

Cytokines are physicochemically diverse, ranging in size from 5 kDa (TGF-α) to 140 kDa (Mullerian-inhibiting substance). They include single polypeptide chains, as well as disulfide-linked homodimers and heterodimers.

Cytokines influence cellular events by binding to cell-surface receptors. Binding initiates a chain of signalling events within the cell, which ultimately results in phenotypic changes such as cell division, protease production, cell migration, expression of cell surface proteins, and production of additional growth factors.

Cell differentiation and maturation are also under control of cytokines. For example, the hematopoietic factors erythropoietin, thrombopoietin, and G-CSF stimulate the production of erythrocytes, platelets, and neutrophils, respectively, from precursor cells in the bone marrow. Development of mature cells from pluripotent progenitors may require the presence of a plurality of factors.

The role of cytokines in controlling cellular processes makes them likely candidates and targets for therapeutic intervention; indeed, a number of cytokines have been approved for clinical use. Interferon-alpha (IFN-α), for example, is used in the treatment of hairy cell leukemia, chronic myeloid leukemia, Kaposi's sarcoma, condylomata acuminata, chronic hepatitis C, and chronic hepatitis B (Aggarwal and Puri, "Common and Uncommon Features of Cytokines and Cytokine Receptors: An Overview", in Aggarwal and Puri, eds., Human Cytokines: Their Role in Disease and Therapy, Blackwell Science, Cambridge, MA, 1995, 3-24). Platelet-derived growth factor (PDGF) has been approved in the United States and other countries for the

treatment of dermal ulcers in diabetic patients. The hematopoietic cytokine erythropoietin has been developed for the treatment of anemias (e.g., EP 613,683). G-CSF, GM-CSF, IFN-β, IFN-γ, and IL-2 have also been approved for use in humans (Aggarwal and Puri, *ibid.*). Experimental evidence supports additional therapeutic uses of cytokines and their inhibitors. Inhibition of PDGF receptor activity has been shown to reduce intimal hyperplasia in injured baboon arteries (Giese et al., Restenosis Summit VIII, Poster Session #23, 1996; U.S. Patent No. 5,620,687). Vascular endothelial growth factors (VEGFs) have been shown to promote the growth of blood vessels in ischemic limbs (Isner et al., *The Lancet* 348:370-374, 1996), and have been proposed for use as wound-healing agents, for treatment of periodontal disease, for promoting endothelialization in vascular graft surgery, and for promoting collateral circulation following myocardial infarction (WIPO Publication No. WO 95/24473; U.S. Patent No. 5,219,739). A soluble VEGF receptor (soluble flt-1) has been found to block binding of VEGF to cell-surface receptors and to inhibit the growth of vascular tissue *in vitro* (*Biotechnology News* 16(17):5-6, 1996). Experimental evidence suggests that inhibition of angiogenesis may be used to block tumor development (*Biotechnology News*, Nov. 13, 1997) and that angiogenesis is an early indicator of cervical cancer (*Br. J. Cancer* 76:1410-1415, 1997). More recently, thrombopoietin has been shown to stimulate the production of platelets *in vivo* (Kaushansky et al., *Nature* 369:568-571, 1994) and has been the subject of several clinical trials (reviewed by von dem Borne et al., *Baillière's Clin. Haematol.* 11:427-445, 1998).

In view of the proven clinical utility of cytokines, there is a need in the art for additional such molecules for use as both therapeutic agents and research tools and reagents. Cytokines are used in the laboratory to study developmental processes, and in laboratory and industry settings as components of cell culture media.

SUMMARY OF THE INVENTION

The present invention provides novel polypeptides, polynucleotides encoding them, and methods of making them, as well as compositions and methods for modulating the proliferation, differentiation, migration, and metabolism of responsive cell types and for regulating tissue development.

Within one aspect of the invention there is provided an isolated polypeptide comprising at least nine contiguous amino acid residues of SEQ ID NO:2. Within one embodiment, the isolated polypeptide of claim 1 consists of from 15 to 1500 amino acid residues. Within another embodiment, the at least nine contiguous amino acid residues of SEQ ID NO:2 are operably linked via a peptide bond or polypeptide linker to a second polypeptide selected from the group consisting of maltose binding protein, an immunoglobulin constant region, a polyhistidine tag, and a peptide as shown in SEQ ID NO:5. Within another embodiment, the isolated polypeptide comprises at least 30 contiguous residues of SEQ ID NO:2. Exemplary polypeptides of the invention include, without limitation, those comprising residues 34-48 of SEQ ID NO:2, residues 49-69 of SEQ ID NO:2, residues 70-84 of SEQ ID NO:2, residues 87-101 of SEQ ID NO:2, residues 102-126 of SEQ ID NO:2, residues 127-141 of SEQ ID NO:2, residues 20-53 of SEQ ID NO:2, residues 49-57 of SEQ ID NO:2, residues 34-147 of SEQ ID NO:3, residues 34-147 of SEQ ID NO:2, residues 34-151 of SEQ ID NO:3, residues 34-151 of SEQ ID NO:2, residues 21-151 of SEQ ID NO:3, and residues 21-151 of SEQ ID NO:2.

Within a second aspect of the invention there is provided an expression vector comprising the following operably linked elements: a transcription promoter; a DNA segment encoding a polypeptide as disclosed above; and a transcription terminator. Within one embodiment, the DNA segment comprises nucleotides 61 to 453 of SEQ ID NO:4. Within another embodiment, the expression vector further comprises a secretory signal sequence operably linked to the DNA segment. An exemplary secretory signal sequence encodes residues 1-20 of SEQ ID NO:2.

Within a third aspect of the invention there is provided a cultured cell into which has been introduced an expression vector as disclosed above, wherein the cell expresses the DNA segment. Within one embodiment, the expression vector comprises a secretory signal sequence operably linked to the DNA segment, and the polypeptide is secreted by the cell. The cultured cell of the invention can be used within a method of making a protein, wherein the cell is cultured under conditions whereby the DNA segment is expressed and the polypeptide is produced, and the produced polypeptide is recovered. Within one embodiment, the expression vector comprises a

secretory signal sequence operably linked to the DNA segment, the polypeptide is secreted by the cell, and the polypeptide is recovered from a medium in which the cell is cultured.

Within a fourth aspect of the invention there is provided a polypeptide produced by the method disclosed above.

With a fifth aspect of the invention there is provided an antibody that specifically binds to a polypeptide as disclosed above.

Within a sixth aspect of the invention there is provided a method of detecting, in a test sample, the presence of an antagonist of zalpha48 activity, comprising the steps of culturing a cell that is responsive to zalpha48, exposing the cell to a zalpha48 polypeptide in the presence and absence of a test sample, comparing levels of response to the zalpha48 polypeptide, in the presence and absence of the test sample, by a biological or biochemical assay, and determining from the comparison the presence of an antagonist of zalpha48 activity in the test sample.

These and other aspects of the invention will become evident upon reference to the following detailed description of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

The figure is a Hopp/Woods hydrophilicity profile of the amino acid sequence shown in SEQ ID NO:2. The profile is based on a sliding six-residue window. Buried G, S, and T residues and exposed H, Y, and W residues were ignored. These residues are indicated in the figure by lower case letters.

DETAILED DESCRIPTION OF THE INVENTION

Prior to setting forth the invention in detail, it may be helpful to the understanding thereof to define the following terms:

The term "affinity tag" is used herein to denote a polypeptide segment that can be attached to a second polypeptide to provide for purification of the second polypeptide or provide sites for attachment of the second polypeptide to a substrate. In principal, any peptide or protein for which an antibody or other specific binding agent is available can be used as an affinity tag. Affinity tags include a poly-histidine tract,

protein A (Nilsson et al., *EMBO J.* 4:1075, 1985; Nilsson et al., *Methods Enzymol.* 198:3, 1991), glutathione S transferase (Smith and Johnson, *Gene* 67:31, 1988), Glu-Glu affinity tag (Grussenmeyer et al., *Proc. Natl. Acad. Sci. USA* 82:7952-4, 1985) (SEQ ID NO:5), substance P, Flag™ peptide (Hopp et al., *Biotechnology* 6:1204-1210, 1988), streptavidin binding peptide, maltose binding protein (Guan et al., *Gene* 67:21-30, 1987), cellulose binding protein, thioredoxin, ubiquitin, T7 polymerase, or other antigenic epitope or binding domain. See, in general, Ford et al., *Protein Expression and Purification* 2: 95-107, 1991. DNAs encoding affinity tags and other reagents are available from commercial suppliers (e.g., Pharmacia Biotech, Piscataway, NJ; New England Biolabs, Beverly, MA; Eastman Kodak, New Haven, CT).

The term "allelic variant" is used herein to denote any of two or more alternative forms of a gene occupying the same chromosomal locus. Allelic variation arises naturally through mutation, and may result in phenotypic polymorphism within populations. Gene mutations can be silent (no change in the encoded polypeptide) or may encode polypeptides having altered amino acid sequence. The term allelic variant is also used herein to denote a protein encoded by an allelic variant of a gene.

The terms "amino-terminal" and "carboxyl-terminal" are used herein to denote positions within polypeptides. Where the context allows, these terms are used with reference to a particular sequence or portion of a polypeptide to denote proximity or relative position. For example, a certain sequence positioned carboxyl-terminal to a reference sequence within a polypeptide is located proximal to the carboxyl terminus of the reference sequence, but is not necessarily at the carboxyl terminus of the complete polypeptide.

A "complement" of a polynucleotide molecule is a polynucleotide molecule having a complementary base sequence and reverse orientation as compared to a reference sequence. For example, the sequence 5' ATGCACGGG 3' is complementary to 5' CCCGTGCAT 3'.

The term "conservative amino acid substitution" refers to a substitution represented by a value of greater than -1 as determined from the BLOSUM62 matrix of Henikoff and Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915-10919, 1992 (Table 1). The BLOSUM62 matrix is an amino acid substitution matrix derived from about 2,000

local multiple alignments of protein sequence segments, representing highly conserved regions of more than 500 groups of related proteins. An amino acid substitution is conservative if the substitution is characterized by a BLOSUM62 value of 0, 1, 2, or 3. Preferred conservative amino acid substitutions are characterized by a BLOSUM62 value of at least 1 (e.g., 1, 2 or 3), while more preferred conservative amino acid substitutions are characterized by a BLOSUM62 value of at least 2 (e.g., 2 or 3).

Table 1

	A	R	N	D	C	Q	E	G	H	I	L	K	M	F	P	S	T	W	Y	V
A	4																			
R	-1	5																		
N	-2	0	6																	
D	-2	-2	1	6																
C	0	-3	-3	-3	9															
Q	-1	1	0	0	-3	5														
E	-1	0	0	2	-4	2	5													
G	0	-2	0	-1	-3	-2	-2	6												
H	-2	0	1	-1	-3	0	0	-2	8											
I	-1	-3	-3	-3	-1	-3	-3	-4	-3	4										
L	-1	-2	-3	-4	-1	-2	-3	-4	-3	2	4									
K	-1	2	0	-1	-3	1	1	-2	-1	-3	-2	5								
M	-1	-1	-2	-3	-1	0	-2	-3	-2	1	2	-1	5							
F	-2	-3	-3	-3	-2	-3	-3	-3	-1	0	0	-3	0	6						
P	-1	-2	-2	-1	-3	-1	-1	-2	-2	-3	-3	-1	-2	-4	7					
S	1	-1	1	0	-1	0	0	0	-1	-2	-2	0	-1	-2	-1	4				
T	0	-1	0	-1	-1	-1	-1	-2	-2	-1	-1	-1	-1	-2	-1	1	5			
W	-3	-3	-4	-4	-2	-2	-3	-2	-2	-3	-2	-3	-1	1	-4	-3	-2	1	1	
Y	-2	-2	-2	-3	-2	-1	-2	-3	2	-1	-1	-2	-1	3	-3	-2	-2	2	7	
V	0	-3	-3	-3	-1	-2	-2	-3	-3	3	1	-2	1	-1	-2	-2	0	-3	-1	4

The term "corresponding to", when applied to positions of amino acid residues in sequences, means corresponding positions in a plurality of sequences when the sequences are optimally aligned.

The term "degenerate nucleotide sequence" denotes a sequence of nucleotides that includes one or more degenerate codons (as compared to a reference polynucleotide molecule that encodes a polypeptide). Degenerate codons contain different triplets of nucleotides, but encode the same amino acid residue (i.e., GAU and GAC triplets each encode Asp).

The term "expression vector" is used to denote a DNA molecule, linear or circular, that comprises a segment encoding a polypeptide of interest operably linked to additional segments that provide for its transcription. Such additional segments include promoter and terminator sequences, and may also include one or more origins of replication, one or more selectable markers, an enhancer, a polyadenylation signal, etc. Expression vectors are generally derived from plasmid or viral DNA, or may contain elements of both.

The term "isolated", when applied to a polynucleotide, denotes that the polynucleotide has been removed from its natural genetic milieu and is thus free of other extraneous or unwanted coding sequences, and is in a form suitable for use within genetically engineered protein production systems. Such isolated molecules are those that are separated from their natural environment and include cDNA and genomic clones. Isolated DNA molecules of the present invention are free of other genes with which they are ordinarily associated, but may include naturally occurring 5' and 3' untranslated regions such as promoters and terminators. The identification of associated regions will be evident to one of ordinary skill in the art (see for example, Dynan and Tijan, *Nature* 316:774-78, 1985).

An "isolated" polypeptide or protein is a polypeptide or protein that is found in a condition other than its native environment, such as apart from blood and animal tissue. The isolated polypeptide or protein can be prepared substantially free of other polypeptides or proteins, particularly those of animal origin. For certain applications it is preferred to provide polypeptides and proteins in a highly purified form, i.e. greater than 95% pure or greater than 99% pure. When used in this context,

the term "isolated" does not exclude the presence of the same polypeptide or protein in alternative physical forms, such as dimers or alternatively glycosylated or derivatized forms.

"Operably linked" means that two or more entities are joined together such that they function in concert for their intended purposes. When referring to DNA segments, the phrase indicates, for example, that coding sequences are joined in the correct reading frame, and transcription initiates in the promoter and proceeds through the coding segment(s) to the terminator. When referring to polypeptides, "operably linked" includes both covalently (e.g., by disulfide bonding) and non-covalently (e.g., by hydrogen bonding, hydrophobic interactions, or salt-bridge interactions) linked sequences, wherein the desired function(s) of the sequences are retained.

The term "ortholog" denotes a polypeptide or protein obtained from one species that is the functional counterpart of a polypeptide or protein from a different species. Sequence differences among orthologs are the result of speciation.

A "polynucleotide" is a single- or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5' to the 3' end. Polynucleotides include RNA and DNA, and may be isolated from natural sources, synthesized *in vitro*, or prepared from a combination of natural and synthetic molecules. Sizes of polynucleotides are expressed as base pairs (abbreviated "bp"), nucleotides ("nt"), or kilobases ("kb"). Where the context allows, the latter two terms may describe polynucleotides that are single-stranded or double-stranded. When the term is applied to double-stranded molecules it is used to denote overall length and will be understood to be equivalent to the term "base pairs". It will be recognized by those skilled in the art that the two strands of a double-stranded polynucleotide may differ slightly in length and that the ends thereof may be staggered as a result of enzymatic cleavage; thus all nucleotides within a double-stranded polynucleotide molecule may not be paired. Such unpaired ends will in general not exceed 20 nt in length.

A "polypeptide" is a polymer of amino acid residues joined by peptide bonds, whether produced naturally or synthetically. Polypeptides of less than about 10 amino acid residues are commonly referred to as "peptides".

The term "promoter" is used herein for its art-recognized meaning to denote a portion of a gene containing DNA sequences that provide for the binding of RNA polymerase and initiation of transcription. Promoter sequences are commonly, but not always, found in the 5' non-coding regions of genes.

A "protein" is a macromolecule comprising one or more polypeptide chains. A protein may also comprise non-peptidic components, such as carbohydrate groups. Carbohydrates and other non-peptidic substituents may be added to a protein by the cell in which the protein is produced, and will vary with the type of cell. Proteins are defined herein in terms of their amino acid backbone structures; substituents such as carbohydrate groups are generally not specified, but may be present nonetheless. thus, a protein "consisting of", for example, from 15 to 1500 amino acid residues may further contain one or more carbohydrate chains.

A "secretory signal sequence" is a DNA sequence that encodes a polypeptide (a "secretory peptide") that, as a component of a larger polypeptide, directs the larger polypeptide through a secretory pathway of a cell in which it is synthesized. The larger polypeptide is commonly cleaved to remove the secretory peptide during transit through the secretory pathway.

A "segment" is a portion of a larger molecule (e.g., polynucleotide or polypeptide) having specified attributes. For example, a DNA segment encoding a specified polypeptide is a portion of a longer DNA molecule, such as a plasmid or plasmid fragment, that, when read from the 5' to the 3' direction, encodes the sequence of amino acids of the specified polypeptide.

Molecular weights and lengths of polymers determined by imprecise analytical methods (e.g., gel electrophoresis) will be understood to be approximate values. When such a value is expressed as "about" X or "approximately" X, the stated value of X will be understood to be accurate to ±10%.

The present invention provides novel cytokine polypeptides and proteins. This novel cytokine, termed "zalpha48", was identified by the presence of polypeptide and polynucleotide features characteristic of four-helix-bundle cytokines (e.g., erythropoietin, thrombopoietin, G-CSF, IL-2, IL-4, leptin, and growth hormone). Analysis of the amino acid sequence shown in SEQ ID NO:2 indicates the presence of

four amphipathic, alpha-helical regions. These regions include at least amino acid residues 34 through 48 (helix A), 70 through 84 (helix B), 87 through 101 (helix C), and 127 through 141 (helix D). Within these helical regions, residues that are expected to lie within the core of the four-helix bundle occur at positions 34, 37, 38, 41, 44, 45, 48, 70, 73, 74, 77, 80, 81, 84, 87, 90, 91, 94, 97, 98, 101, 127, 130, 131, 134, 137, 138, and 141 of SEQ ID NO:2. Residues 35, 36, 39, 40, 42, 43, 46, 47, 71, 72, 75, 76, 78, 79, 82, 83, 88, 89, 92, 93, 95, 96, 99, 100, 128, 129, 132, 133, 135, 136, 139, and 140 are expected to lie on the exposed surface of the bundle. Inter-helix loops comprise approximately residues 49 through 69 (loop A-B) and 102 through 126 (loop C-D). The human zalpha48 cDNA (SEQ ID NO:1) encodes a polypeptide of 151 amino acid residues. While not wishing to be bound by theory, this sequence is predicted to include a secretory peptide of 20 residues. Cleavage after residue 20 will result in a mature polypeptide (residues 21-151 of SEQ ID NO:2) having a calculated molecular weight (exclusive of glycosylation) of 14,823 Da. Those skilled in the art will recognize, however, that some cytokines (e.g., endothelial cell growth factor, basic FGF, and IL-1β) do not comprise conventional secretory peptides and are secreted by a mechanism that is not understood. The cDNA also includes a clear polyadenylation signal, as well as two message instability motifs (ATTTA) in the 3'-untranslated region beginnning at nucleotides 749 and 862 of SEQ ID NO:1. These message instability motifs are characteristic of cytokine genes (see, e.g., Shaw and Kamen, *Cell* 46:659-667, 1986).

Those skilled in the art will recognize that predicted domain boundaries are somewhat imprecise and may vary by up to ± 5 amino acid residues.

Polypeptides of the present invention comprise at least 6, at least 9, or at least 15 contiguous amino acid residues of SEQ ID NO:2. Within certain embodiments of the invention, the polypeptides comprise 20, 30, 40, 50, 100, or more contiguous residues of SEQ ID NO:2, up to the entire predicted mature polypeptide (residues 21 to 151 of SEQ ID NO:2) or the primary translation product (residues 1 to 151 of SEQ ID NO:2). As disclosed in more detail below, these polypeptides can further comprise additional, non-zalpha48, polypeptide sequence(s).

Within the polypeptides of the present invention are polypeptides that comprise an epitope-bearing portion of a protein as shown in SEQ ID NO:2. An

"epitope" is a region of a protein to which an antibody can bind. See, for example, Geysen et al., *Proc. Natl. Acad. Sci. USA* 81:3998-4002, 1984. Epitopes can be linear or conformational, the latter being composed of discontinuous regions of the protein that form an epitope upon folding of the protein. Linear epitopes are generally at least 6 amino acid residues in length. Relatively short synthetic peptides that mimic part of a protein sequence are routinely capable of eliciting an antiserum that reacts with the partially mimicked protein. See, Sutcliffe et al., *Science* 219:660-666, 1983. Antibodies that recognize short, linear epitopes are particularly useful in analytic and diagnostic applications that employ denatured protein, such as Western blotting (Tobin, *Proc. Natl. Acad. Sci. USA* 76:4350-4356, 1979), or in the analysis of fixed cells or tissue samples. Antibodies to linear epitopes are also useful for detecting fragments of zalpha48, such as might occur in body fluids or cell culture media.

Antigenic, epitope-bearing polypeptides of the present invention are useful for raising antibodies, including monoclonal antibodies, that specifically bind to a zalpha48 protein. Antigenic, epitope-bearing polypeptides contain a sequence of at least six, often at least nine, more commonly from 15 to about 30 contiguous amino acid residues of a zalpha48 protein (e.g., SEQ ID NO:2). Polypeptides comprising a larger portion of a zalpha48 protein, i.e. from 30 to 50 residues up to the entire sequence, are included. It is preferred that the amino acid sequence of the epitope-bearing polypeptide is selected to provide substantial solubility in aqueous solvents, that is the sequence includes relatively hydrophilic residues, and hydrophobic residues are substantially avoided. Such regions include the interdomain loops of zalpha48 and fragments thereof. Specific polypeptides in this regard include those comprising residues 2-7, 20-33, 20-34, 25-30, 46-51, 47-57, 49-57, 89-94, and 146-151 of SEQ ID NO:2.

Of particular interest within the present invention are polypeptides that comprise the entire four-helix bundle of a zalpha48 polypeptide (e.g., residues 34-141 of SEQ ID NO:2). Such polypeptides may further comprise all or part of one or both of the native zalpha48 amino-terminal (residues 21-33 of SEQ ID NO:2) and carboxyl-terminal (residues 142-151 of SEQ ID NO:2) regions, as well as non-zalpha48 amino acid residues or polypeptide sequences as disclosed in more detail below.

Polypeptides of the present invention can be prepared with one or more amino acid substitutions, deletions or additions as compared to SEQ ID NO:2. Where preservation of zalpha48 biological activity is desired, these changes will ordinarily be of a minor nature, that is conservative amino acid substitutions and other changes that do not significantly affect the folding or activity of the protein or polypeptide. Sequence changes in this regard include amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue, an amino or carboxyl-terminal cysteine residue to facilitate subsequent linking to maleimide-activated keyhole limpet hemocyanin, a linker peptide (typically, but not exclusively, of up to about 20-25 residues), or an extension that facilitates purification (an affinity tag) as disclosed above. Two or more affinity tags may be used in combination. Polypeptides comprising affinity tags can further comprise a polypeptide linker and/or a proteolytic cleavage site between the zalpha48 polypeptide and the affinity tag. Exemplary cleavage sites include thrombin cleavage sites and factor Xa cleavage sites.

The present invention further provides a variety of other polypeptide fusions. For example, a zalpha48 polypeptide can be prepared as a fusion to a dimerizing protein as disclosed in U.S. Patents Nos. 5,155,027 and 5,567,584. Exemplary dimerizing proteins in this regard include immunoglobulin constant region domains. Immunoglobulin-zalpha48 polypeptide fusions can be expressed in genetically engineered cells to produce a variety of multimeric zalpha48 analogs. In addition, a zalpha48 polypeptide can be joined to another bioactive molecule, such as a cytokine, to provide a multi-functional molecule. One or more helices of a zalpha48 polypeptide can be joined to another cytokine to enhance or otherwise modify its biological properties. Auxiliary domains can be fused to zalpha48 polypeptides to target them to specific cells, tissues, or macromolecules (e.g., collagen). For example, a zalpha48 polypeptide or protein can be targeted to a predetermined cell type by fusing a zalpha48 polypeptide to a ligand that specifically binds to a receptor on the surface of the target cell. In this way, polypeptides and proteins can be targeted for therapeutic or diagnostic purposes. A zalpha48 polypeptide can be fused to two or more moieties, such as an affinity tag for purification and a targeting domain. Polypeptide fusions can

also comprise one or more cleavage sites, particularly between domains. See, Tuan et al., *Connective Tissue Research* 34:1-9, 1996.

Polypeptide fusions of the present invention will generally contain not more than about 1,500 amino acid residues, often not more than about 1,200 residues, commonly not more than about 1,000 residues, and will in many cases be considerably smaller. For example, a zalpha48 polypeptide of 131 residues (residues 21-151 of SEQ ID NO:2) can be fused to *E. coli* β-galactosidase (1,021 residues; see Casadaban et al., *J. Bacteriol.* 143:971-980, 1980), a 10-residue spacer, and a 4-residue factor Xa cleavage site to yield a polypeptide of 1,166 residues. In a second example, residues 21-151 of SEQ ID NO:2 can be fused to maltose binding protein (approximately 370 residues), a 4-residue cleavage site, and a 6-residue polyhistidine tag.

The polypeptidews of the present invention can comprise non-naturally occuring amino acid residues. Non-naturally occuring amino acids include, without limitation, *trans*-3-methylproline, 2,4-methanoproline, *cis*-4-hydroxyproline, *trans*-4-hydroxyproline, *N*-methylglycine, *allo*-threonine, methylthreonine, hydroxyethylcysteine, hydroxyethylhomocysteine, nitroglutamine, homoglutamine, pipecolic acid, *tert*-leucine, norvaline, 2-azaphenylalanine, 3-azaphenylalanine, 4-azaphenylalanine, and 4-fluorophenylalanine. Several methods are known in the art for incorporating non-naturally occuring amino acid residues into proteins. See, for example, Robertson et al., *J. Am. Chem. Soc.* 113:2722, 1991; Ellman et al., *Methods Enzymol.* 202:301, 1991; Chung et al., *Science* 259:806-809, 1993; Chung et al., *Proc. Natl. Acad. Sci. USA* 90:10145-10149, 1993; Turcatti et al., *J. Biol. Chem.* 271:19991-19998, 1996; and Koide et al., *Biochem.* 33:7470-7476, 1994. Chemical modification can be combined with site-directed mutagenesis to further expand the range of substitutions (Wynn and Richards, *Protein Sci.* 2:395-403, 1993).

Amino acid sequence changes are made in zalpha48 polypeptides so as to minimize disruption of higher order structure essential to biological activity. Amino acid residues that are within regions or domains that are critical to maintaining structural integrity can be determined. Within these regions one can identify specific residues that will be more or less tolerant of change and maintain the overall tertiary structure of the molecule. Methods for analyzing sequence structure include, but are not

limited to, alignment of multiple sequences with high amino acid or nucleotide identity, secondary structure propensities, binary patterns, complementary packing, and buried polar interactions (Barton, *Current Opin. Struct. Biol.* 5:372-376, 1995 and Cordes et al., *Current Opin. Struct. Biol.* 6:3-10, 1996). In general, determination of structure will be accompanied by evaluation of activity of modified molecules. For example, changes in amino acid residues will be made so as not to disrupt the four-helix bundle structure of the protein family. The effects of amino acid sequence changes can be predicted by, for example, computer modeling using available software (e.g., the Insight II® viewer and homology modeling tools; MSI, San Diego, CA) or determined by analysis of crystal structure (see, e.g., Lapthorn et al, *Nature* 369:455-461, 1994; Lapthorn et al., *Nat. Struct. Biol.* 2:266-268, 1995). Protein folding can be measured by circular dichroism (CD). Measuring and comparing the CD spectra generated by a modified molecule and standard molecule are routine in the art (Johnson, *Proteins* 7:205-214, 1990). Crystallography is another well known and accepted method for analyzing folding and structure. Nuclear magnetic resonance (NMR), digestive peptide mapping and epitope mapping are other known methods for analyzing folding and structural similarities between proteins and polypeptides (Schaanan et al., *Science* 257:961-964, 1992). Mass spectrometry and chemical modification using reduction and alkylation can be used to identify cysteine residues that are associated with disulfide bonds or are free of such associations (Bean et al., *Anal. Biochem.* 201:216-226, 1992; Gray, *Protein Sci.* 2:1732-1748, 1993; and Patterson et al., *Anal. Chem.* 66:3727-3732, 1994). Alterations in disulfide bonding will be expected to affect protein folding. These techniques can be employed individually or in combination to analyze and compare the structural features that affect folding of a variant protein or polypeptide to a standard molecule to determine whether such modifications would be significant.

A hydrophilicity profile of SEQ ID NO:2 is shown in the attached figure. Those skilled in the art will recognize that this hydrophilicity will be taken into account when designing alterations in the amino acid sequence of a zalpha48 polypeptide, so as not to disrupt the overall profile. Residues within the core of the four-helix bundle can be replaced with a hydrophobic residue selected from the group consisting of Leu, Ile, Val, Met, Phe, Trp, Gly, and Ala as shown in SEQ ID NO:3. Cysteine residues at

positions 66, 111, 125, and 147 of SEQ ID NO:2 and the residues predicted to be on the exposed surface of the four-helix bundle will be relatively intolerant of substitution. The length and amino acid composition of the interdomain loops are also expected to be important for receptor binding (and therefore biological activity); conservative substitutions and relatively small insertions and deletions are thus preferred within the loops, and the insertion of bulky amino acid residues (e.g., Phe) will in general be avoided. Zalpha48 variant proteins having amino acid substitutions within the four-helix bundle are shown in SEQ ID NO:3.

Essential amino acids in the polypeptides of the present invention can be identified experimentally according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, *Science* 244, 1081-1085, 1989; Bass et al., *Proc. Natl. Acad. Sci. USA* 88:4498-4502, 1991). In the latter technique, single alanine mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for biological activity as disclosed below to identify amino acid residues that are critical to the activity of the molecule.

Multiple amino acid substitutions can be made and tested using known methods of mutagenesis and screening, such as those disclosed by Reidhaar-Olson and Sauer (*Science* 241:53-57, 1988) or Bowie and Sauer (*Proc. Natl. Acad. Sci. USA* 86:2152-2156, 1989). Briefly, these authors disclose methods for simultaneously randomizing two or more positions in a polypeptide, selecting for functional polypeptide, and then sequencing the mutagenized polypeptides to determine the spectrum of allowable substitutions at each position. Other methods that can be used include phage display (e.g., Lowman et al., *Biochem.* 30:10832-10837, 1991; Ladner et al., U.S. Patent No. 5,223,409; Huse, WIPO Publication WO 92/06204) and region-directed mutagenesis (Derbyshire et al., *Gene* 46:145, 1986; Ner et al., *DNA* 7:127, 1988).

Variants of the disclosed zalpha48 DNA and polypeptide sequences can be generated through DNA shuffling as disclosed by Stemmer, *Nature* 370:389-391, 1994 and Stemmer, *Proc. Natl. Acad. Sci. USA* 91:10747-10751, 1994. Briefly, variant genes are generated by *in vitro* homologous recombination by random fragmentation of

a parent gene followed by reassembly using PCR, resulting in randomly introduced point mutations. This technique can be modified by using a family of parent genes, such as allelic variants or genes from different species, to introduce additional variability into the process. Selection or screening for the desired activity, followed by additional iterations of mutagenesis and assay provides for rapid "evolution" of sequences by selecting for desirable mutations while simultaneously selecting against detrimental changes.

In many cases, the structure of the final polypeptide product will result from processing of the nascent polypeptide chain by the host cell, thus the final sequence of a zalpha48 polypeptide produced by a host cell will not always correspond to the full sequence encoded by the expressed polynucleotide. For example, expressing the complete zalpha48 sequence in a cultured mammalian cell is expected to result in removal of at least the secretory peptide, while the same polypeptide produced in a prokaryotic host would not be expected to be cleaved. Differential processing of individual chains may result in heterogeneity of expressed polypeptides.

The human zalpha48 polypeptide sequence (SEQ ID NO:2) contains five cysteine residues, at positions 66, 111, 125, 134, and 147. Structural predictions indicate that cys residues 66 and 147 may form an intrachain disulfide bond, and that residues 111 and 125 may be free to form interchain disulfide bonds, resulting in dimerization. Actual conformation will depend in part upon the cell in which in the polypeptide is expressed. The polypeptides of the present invention thus include those comprising these cysteine residues, such as polypeptides comprising residues 34-147 of SEQ ID NO:2.

Zalpha48 proteins of the present invention are characterized by their activity, that is, modulation of the proliferation, differentiation, migration, adhesion, or metabolism of responsive cell types. Biological activity of zalpha48 proteins is assayed using *in vitro* or *in vivo* assays designed to detect cell proliferation, differentiation, migration or adhesion; or changes in cellular metabolism (e.g., production of other growth factors or other macromolecules). Many suitable assays are known in the art, and representative assays are disclosed herein. Assays using cultured cells are most convenient for screening, such as for determining the effects of amino acid

substitutions, deletions, or insertions. However, in view of the complexity of developmental processes (e.g., angiogenesis, wound healing), *in vivo* assays will generally be employed to confirm and further characterize biological activity. Certain *in vitro* models, such as the three-dimensional collagen gel matrix model of Pepper et al. (*Biochem. Biophys. Res. Comm.* 189:824-831, 1992), are sufficiently complex to assay histological effects. Assays can be performed using exogenously produced proteins, or may be carried out *in vivo* or *in vitro* using cells expressing the polypeptide(s) of interest. Assays can be conducted using zalpha48 proteins alone or in combination with other growth factors, such as members of the VEGF family or hematopoietic cytokines (e.g., EPO, TPO, G-CSF, stem cell factor). Representative assays are disclosed below.

Mutagenesis methods as disclosed above can be combined with high volume or high-throughput screening methods to detect biological activity of zalpha48 variant polypeptides. Assays that can be scaled up for high throughput include mitogenesis assays, which can be run in a 96-well format. Mutagenized DNA molecules that encode active zalpha48 polypeptides can be recovered from the host cells and rapidly sequenced using modern equipment. These methods allow the rapid determination of the importance of individual amino acid residues in a polypeptide of interest, and can be applied to polypeptides of unknown structure.

Using the methods discussed above, one of ordinary skill in the art can prepare a variety of polypeptide fragments or variants of SEQ ID NO:2 that retain the activity of wild-type zalpha48.

The present invention also provides polynucleotide molecules, including DNA and RNA molecules, that encode the zalpha48 polypeptides disclosed above. A representative DNA sequence encoding the amino acid sequence of SEQ ID NO:2 is shown in SEQ ID NO:1. Those skilled in the art will readily recognize that, in view of the degeneracy of the genetic code, considerable sequence variation is possible among these polynucleotide molecules. SEQ ID NO:4 is a degenerate DNA sequence that encompasses all DNAs that encode the zalpha48 polypeptide of SEQ ID NO: 2. Those skilled in the art will recognize that the degenerate sequence of SEQ ID NO:4 also provides all RNA sequences encoding SEQ ID NO:2 by substituting U for T. Thus,

zalpha48 polypeptide-encoding polynucleotides comprising nucleotides 1-453 or nucleotides 61-453 of SEQ ID NO:4, and their RNA equivalents are contemplated by the present invention, as are segments of SEQ ID NO:4 encoding other zalpha48 polypeptides disclosed herein. Table 2 sets forth the one-letter codes used within SEQ ID NO:4 to denote degenerate nucleotide positions. "Resolutions" are the nucleotides denoted by a code letter. "Complement" indicates the code for the complementary nucleotide(s). For example, the code Y denotes either C or T, and its complement R denotes A or G, A being complementary to T, and G being complementary to C.

TABLE 2

Nucleotide	Resolutions	Complement	Resolutions
A	A	T	T
C	C	G	G
G	G	C	C
T	T	A	A
R	A|G	Y	C|T
Y	C|T	R	A|G
M	A|C	K	G|T
K	G|T	M	A|C
S	C|G	S	C|G
W	A|T	W	A|T
H	A|C|T	D	A|G|T
B	C|G|T	V	A|C|G
V	A|C|G	B	C|G|T
D	A|G|T	H	A|C|T
N	A|C|G|T	N	A|C|G|T

The degenerate codons used in SEQ ID NO:4, encompassing all possible codons for a given amino acid, are set forth in Table 3, below.

TABLE 3

Amino Acid	One-Letter Code	Codons	Degenerate Codon
Cys	C	TGC TGT	TGY
Ser	S	AGC AGT TCA TCC TCG TCT	WSN
Thr	T	ACA ACC ACG ACT	CAN
Pro	P	CCA CCC CCG CCT	CCN
Ala	A	GCA GCC GCG GCT	GCN
Gly	G	GGA GGC GGG GGT	GGN
Asn	N	AAC AAT	AAY
Asp	D	GAC GAT	GAY
Glu	E	GAA GAG	GAR
Gln	Q	CAA CAG	CAR
His	H	CAC CAT	CAY
Arg	R	AGA AGG CGA CGC CGG CGT	MGN
Lys	K	AAA AAG	AAR
Met	M	ATG	ATG
Ile	I	ATA ATC ATT	ATH
Leu	L	CTA CTC CTG CTT TTA TTG	YTN
Val	V	GTA GTC GTG GTT	GTN
Phe	F	TTC TTT	TTY
Tyr	Y	TAC TAT	TAY
Trp	W	TGG	TGG
Ter	.	TAA TAG TGA	TRR
Asn|Asp	B		RAY
Glu|Gln	Z		SAR
Any	X		NNN
Gap	-	---	

One of ordinary skill in the art will appreciate that some ambiguity is introduced in determining a degenerate codon, representative of all possible codons encoding each amino acid. For example, the degenerate codon for serine (WSN) can, in some circumstances, encode arginine (AGR), and the degenerate codon for arginine (MGN) can, in some circumstances, encode serine (AGY). A similar relationship exists between codons encoding phenylalanine and leucine. Thus, some polynucleotides encompassed by the degenerate sequence may encode variant amino acid sequences, but one of ordinary skill in the art can easily identify such variant sequences by reference to the amino acid sequence of SEQ ID NO: 2. Variant sequences can be readily tested for functionality as described herein.

One of ordinary skill in the art will also appreciate that different species can exhibit preferential codon usage. See, in general, Grantham et al., *Nuc. Acids Res.* 8:1893-912, 1980; Haas et al. *Curr. Biol.* 6:315-24, 1996; Wain-Hobson et al., *Gene* 13:355-64, 1981; Grosjean and Fiers, *Gene* 18:199-209, 1982; Holm, *Nuc. Acids Res.* 14:3075-87, 1986; and Ikemura, *J. Mol. Biol.* 158:573-97, 1982. Introduction of preferred codon sequences into recombinant DNA can, for example, enhance production of the protein by making protein translation more efficient within a particular cell type or species. Therefore, the degenerate codon sequence disclosed in SEQ ID NO:4 serves as a template for optimizing expression of polynucleotides in various cell types and species commonly used in the art and disclosed herein.

Within certain embodiments of the invention the isolated polynucleotides will hybridize to similar sized regions of SEQ ID NO:1 or a sequence complementary thereto under stringent conditions. In general, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Typical stringent conditions are those in which the salt concentration is up to about 0.03 M at pH 7 and the temperature is at least about 60°C.

As previously noted, the isolated polynucleotides of the present invention include DNA and RNA. Methods for preparing DNA and RNA are well known in the art. In general, RNA is isolated from a tissue or cell that produces large

amounts of zalpha48 RNA. Pancreatic islet cells are preferred. Fibroblasts are another preferred source. Total RNA can be prepared using guanidine HCl extraction followed by isolation by centrifugation in a CsCl gradient (Chirgwin et al., *Biochemistry* 18:52-94, 1979). Poly $(A)^+$ RNA is prepared from total RNA using the method of Aviv and Leder (*Proc. Natl. Acad. Sci. USA* 69:1408-1412, 1972). Complementary DNA (cDNA) is prepared from poly$(A)^+$ RNA using known methods. In the alternative, genomic DNA can be isolated. Polynucleotides encoding zalpha48 polypeptides are then identified and isolated by, for example, hybridization or PCR.

Full-length clones encoding zalpha48 can be obtained by conventional cloning procedures. Complementary DNA (cDNA) clones are generally preferred, although for some applications (e.g., expression in transgenic animals) it may be preferable to use a genomic clone, or to modify a cDNA clone to include at least one genomic intron. Methods for preparing cDNA and genomic clones are well known and within the level of ordinary skill in the art, and include the use of the sequence disclosed herein, or parts thereof, for probing or priming a library. Expression libraries can be probed with antibodies to zalpha48, receptor fragments, or other specific binding partners.

Zalpha48 polynucleotide sequences disclosed herein can also be used as probes or primers to clone 5' non-coding regions of a zalpha48 gene. Promoter elements from a zalpha48 gene can thus be used to direct the expression of heterologous genes in, for example, transgenic animals or patients treated with gene therapy. Cloning of 5' flanking sequences also facilitates production of zalpha48 proteins by "gene activation" as disclosed in U.S. Patent No. 5,641,670. Briefly, expression of an endogenous zalpha48 gene in a cell is altered by introducing into the zalpha48 locus a DNA construct comprising at least a targeting sequence, a regulatory sequence, an exon, and an unpaired splice donor site. The targeting sequence is a zalpha48 5' non-coding sequence that permits homologous recombination of the construct with the endogenous zalpha48 locus, whereby the sequences within the construct become operably linked with the endogenous zalpha48 coding sequence. In this way, an endogenous zalpha48 promoter can be replaced or supplemented with other regulatory sequences to provide enhanced, tissue-specific, or otherwise regulated expression.

Those skilled in the art will recognize that the sequences disclosed in SEQ ID NOS:1 and 2 represent a single allele of human zalpha48. Allelic variants of these sequences can be cloned by probing cDNA or genomic libraries from different individuals according to standard procedures.

The present invention further provides counterpart polypeptides and polynucleotides from other species ("orthologs"). Of particular interest are zalpha48 polypeptides from other mammalian species, including murine, porcine, ovine, bovine, canine, feline, equine, and other primate polypeptides. Orthologs of human zalpha48 can be cloned using information and compositions provided by the present invention in combination with conventional cloning techniques. For example, a cDNA can be cloned using mRNA obtained from a tissue or cell type that expresses zalpha48 as disclosed above. A library is then prepared from mRNA of a positive tissue or cell line. A zalpha48-encoding cDNA can then be isolated by a variety of methods, such as by probing with a complete or partial human cDNA or with one or more sets of degenerate probes based on the disclosed sequence. A cDNA can also be cloned using the polymerase chain reaction, or PCR (Mullis, U.S. Patent No. 4,683,202), using primers designed from the representative human zalpha48 sequence disclosed herein. Within an additional method, the cDNA library can be used to transform or transfect host cells, and expression of the cDNA of interest can be detected with an antibody to zalpha48 polypeptide. Similar techniques can also be applied to the isolation of genomic clones.

For any zalpha48 polypeptide, including variants and fusion proteins, one of ordinary skill in the art can readily generate a fully degenerate polynucleotide sequence encoding that variant using the information set forth in Tables 2 and 3, above. Moreover, those of skill in the art can use standard software to devise zalpha48 variants based upon the nucleotide and amino acid sequences described herein. The present invention thus provides a computer-readable medium encoded with a data structure that provides at least one of the following sequences: SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, and portions thereof. Suitable forms of computer-readable media include magnetic media and optically-readable media. Examples of magnetic media include a hard or fixed drive, a random access memory (RAM) chip, a floppy disk, digital linear tape (DLT), a disk cache, and a ZIP™ disk. Optically readable media are

exemplified by compact discs (e.g., CD-read only memory (ROM), CD-rewritable (RW), and CD-recordable), and digital versatile/video discs (DVD) (e.g., DVD-ROM, DVD-RAM, and DVD+RW).

The zalpha48 polypeptides of the present invention, including full-length polypeptides, biologically active fragments, and fusion polypeptides can be produced according to conventional techniques using cells into which have been introduced an expression vector encoding the polypeptide. As used herein, "cells into which have been introduced an expression vector" include both cells that have been directly manipulated by the introduction of exogenous DNA molecules and progeny thereof that contain the introduced DNA. Suitable host cells are those cell types that can be transformed or transfected with exogenous DNA and grown in culture, and include bacteria, fungal cells, and cultured higher eukaryotic cells. Techniques for manipulating cloned DNA molecules and introducing exogenous DNA into a variety of host cells are disclosed by Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989, and Ausubel et al., eds., *Current Protocols in Molecular Biology*, John Wiley and Sons, Inc., NY, 1987.

In general, a DNA sequence encoding a zalpha48 polypeptide is operably linked to other genetic elements required for its expression, generally including a transcription promoter and terminator, within an expression vector. The vector will also commonly contain one or more selectable markers and one or more origins of replication, although those skilled in the art will recognize that within certain systems selectable markers may be provided on separate vectors, and replication of the exogenous DNA may be provided by integration into the host cell genome. Selection of promoters, terminators, selectable markers, vectors and other elements is a matter of routine design within the level of ordinary skill in the art. Many such elements are described in the literature and are available through commercial suppliers.

To direct a zalpha48 polypeptide into the secretory pathway of a host cell, a secretory signal sequence (also known as a leader sequence, prepro sequence or pre sequence) is provided in the expression vector. The secretory signal sequence may be that of zalpha48, or may be derived from another secreted protein (e.g., t-PA; see, U.S. Patent No. 5,641,655) or synthesized *de novo*. The secretory signal sequence is

operably linked to the zalpha48 DNA sequence, i.e., the two sequences are joined in the correct reading frame and positioned to direct the newly sythesized polypeptide into the secretory pathway of the host cell. Secretory signal sequences are commonly positioned 5' to the DNA sequence encoding the polypeptide of interest, although certain signal sequences may be positioned elsewhere in the DNA sequence of interest (see, e.g., Welch et al., U.S. Patent No. 5,037,743; Holland et al., U.S. Patent No. 5,143,830).

Cultured mammalian cells can be used as hosts within the present invention. Methods for introducing exogenous DNA into mammalian host cells include calcium phosphate-mediated transfection (Wigler et al., *Cell* 14:725, 1978; Corsaro and Pearson, *Somatic Cell Genetics* 7:603, 1981; Graham and Van der Eb, *Virology* 52:456, 1973), electroporation (Neumann et al., *EMBO J.* 1:841-845, 1982), DEAE-dextran mediated transfection (Ausubel et al., ibid.), and liposome-mediated transfection (Hawley-Nelson et al., *Focus* 15:73, 1993; Ciccarone et al., *Focus* 15:80, 1993). The production of recombinant polypeptides in cultured mammalian cells is disclosed, for example, by Levinson et al., U.S. Patent No. 4,713,339; Hagen et al., U.S. Patent No. 4,784,950; Palmiter et al., U.S. Patent No. 4,579,821; and Ringold, U.S. Patent No. 4,656,134. Suitable cultured mammalian cells include the COS-1 (ATCC No. CRL 1650), COS-7 (ATCC No. CRL 1651), BHK (ATCC No. CRL 1632), BHK 570 (ATCC No. CRL 10314), 293 (ATCC No. CRL 1573; Graham et al., *J. Gen. Virol.* 36:59-72, 1977) and Chinese hamster ovary (e.g. CHO-K1, ATCC No. CCL 61; or CHO DG44, Chasin et al., *Som. Cell. Molec. Genet.* 12:555, 1986) cell lines. Additional suitable cell lines are known in the art and available from public depositories such as the American Type Culture Collection, Manassas, VA. Suitable promoters include those from SV-40 or cytomegalovirus (e.g., U.S. Patent No. 4,956,288), metallothionein genes (U.S. Patent Nos. 4,579,821 and 4,601,978), and the adenovirus major late promoter. Expression vectors for use in mammalian cells include pZP-1 and pZP-9, which have been deposited with the American Type Culture Collection, Manassas, VA USA under accession numbers 98669 and 98668, respectively, and derivatives thereof.

Drug selection is generally used to select for cultured mammalian cells into which foreign DNA has been inserted. Such cells are commonly referred to as "transfectants". Cells that have been cultured in the presence of the selective agent and

are able to pass the gene of interest to their progeny are referred to as "stable transfectants." An exemplary selectable marker is a gene encoding resistance to the antibiotic neomycin. Selection is carried out in the presence of a neomycin-type drug, such as G-418 or the like. Selection systems can also be used to increase the expression level of the gene of interest, a process referred to as "amplification." Amplification is carried out by culturing transfectants in the presence of a low level of the selective agent and then increasing the amount of selective agent to select for cells that produce high levels of the products of the introduced genes. An exemplary amplifiable selectable marker is dihydrofolate reductase, which confers resistance to methotrexate. Other drug resistance genes (e.g. hygromycin resistance, multi-drug resistance, puromycin acetyltransferase) can also be used.

The adenovirus system (disclosed in more detail below) can also be used for protein production *in vitro*. By culturing adenovirus-infected non-293 cells under conditions where the cells are not rapidly dividing, the cells can produce proteins for extended periods of time. For instance, BHK cells are grown to confluence in cell factories, then exposed to the adenoviral vector encoding the secreted protein of interest. The cells are then grown under serum-free conditions, which allows infected cells to survive for several weeks without significant cell division. In an alternative method, adenovirus vector-infected 293 cells can be grown as adherent cells or in suspension culture at relatively high cell density to produce significant amounts of protein (See Garnier et al., *Cytotechnol.* 15:145-55, 1994). With either protocol, an expressed, secreted heterologous protein can be repeatedly isolated from the cell culture supernatant, lysate, or membrane fractions depending on the disposition of the expressed protein in the cell. Within the infected 293 cell production protocol, non-secreted proteins can also be effectively obtained.

Insect cells can be infected with recombinant baculovirus, commonly derived from *Autographa californica* nuclear polyhedrosis virus (AcNPV) according to methods known in the art. Within one method, recombinant baculovirus is produced through the use of a transposon-based system described by Luckow et al. (*J. Virol.* 67:4566-4579, 1993). This system, which utilizes transfer vectors, is commercially available in kit form (Bac-to-Bac™ kit; Life Technologies, Rockville, MD). The

transfer vector (e.g., pFastBac1™; Life Technologies) contains a Tn7 transposon to move the DNA encoding the protein of interest into a baculovirus genome maintained in *E. coli* as a large plasmid called a "bacmid." See, Hill-Perkins and Possee, *J. Gen. Virol.* 71:971-976, 1990; Bonning et al., *J. Gen. Virol.* 75:1551-1556, 1994; and Chazenbalk and Rapoport, *J. Biol. Chem.* 270:1543-1549, 1995. For protein production, the recombinant virus is used to infect host cells, typically a cell line derived from the fall armyworm, *Spodoptera frugiperda* (e.g., Sf9 or Sf21 cells) or *Trichoplusia ni* (e.g., High Five™ cells; Invitrogen, Carlsbad, CA). See, for example, U.S. Patent No. 5,300,435. Serum-free media are used to grow and maintain the cells. Suitable media formulations are known in the art and can be obtained from commercial suppliers. The cells are grown up from an inoculation density of approximately 2-5 x 10^5 cells to a density of 1-2 x 10^6 cells, at which time a recombinant viral stock is added at a multiplicity of infection (MOI) of 0.1 to 10, more typically near 3. Procedures used are generally known in the art.

Other higher eukaryotic cells can also be used as hosts, including plant cells and avian cells. The use of *Agrobacterium rhizogenes* as a vector for expressing genes in plant cells has been reviewed by Sinkar et al., *J. Biosci. (Bangalore)* 11:47-58, 1987.

Fungal cells, including yeast cells, can also be used within the present invention. Yeast species of particular interest in this regard include *Saccharomyces cerevisiae*, *Pichia pastoris*, and *Pichia methanolica*. Methods for transforming *S. cerevisiae* cells with exogenous DNA and producing recombinant polypeptides therefrom are disclosed by, for example, Kawasaki, U.S. Patent No. 4,599,311; Kawasaki et al., U.S. Patent No. 4,931,373; Brake, U.S. Patent No. 4,870,008; Welch et al., U.S. Patent No. 5,037,743; and Murray et al., U.S. Patent No. 4,845,075. Transformed cells are selected by phenotype determined by the selectable marker, such as drug resistance, the ability to grow in the absence of a particular nutrient (e.g., leucine), or the *POT1* vector system disclosed by Kawasaki et al. (U.S. Patent No. 4,931,373), which allows transformed cells to be selected by growth in glucose-containing media. Suitable promoters and terminators for use in yeast include those from glycolytic enzyme genes (see, e.g., Kawasaki, U.S. Patent No. 4,599,311;

Kingsman et al., U.S. Patent No. 4,615,974; and Bitter, U.S. Patent No. 4,977,092) and alcohol dehydrogenase genes. See also U.S. Patents Nos. 4,990,446; 5,063,154; 5,139,936 and 4,661,454. Transformation systems for other yeasts, including *Hansenula polymorpha, Schizosaccharomyces pombe, Kluyveromyces lactis, Kluyveromyces fragilis, Ustilago maydis, Pichia pastoris, Pichia methanolica, Pichia guillermondii* and *Candida maltosa* are known in the art. See, for example, Gleeson et al., *J. Gen. Microbiol.* 132:3459-3465, 1986; Cregg, U.S. Patent No. 4,882,279; and Raymond et al., *Yeast* 14, 11-23, 1998. Aspergillus cells may be utilized according to the methods of McKnight et al., U.S. Patent No. 4,935,349. Methods for transforming *Acremonium chrysogenum* are disclosed by Sumino et al., U.S. Patent No. 5,162,228. Methods for transforming Neurospora are disclosed by Lambowitz, U.S. Patent No. 4,486,533. Production of recombinant proteins in *Pichia methanolica* is disclosed in U.S. Patents No. 5,716,808, 5,736,383, 5,854,039, and 5,888,768.

Prokaryotic host cells, including strains of the bacteria *Escherichia coli*, *Bacillus* and other genera are also useful host cells within the present invention. Techniques for transforming these hosts and expressing foreign DNA sequences cloned therein are well known in the art (see, e.g., Sambrook et al., *ibid.*). When expressing a zalpha48 polypeptide in bacteria such as *E. coli*, the polypeptide may be retained in the cytoplasm, typically as insoluble granules, or may be directed to the periplasmic space by a bacterial secretion sequence. In the former case, the cells are lysed, and the granules are recovered and denatured using, for example, guanidine isothiocyanate or urea. The denatured polypeptide can then be refolded and dimerized by diluting the denaturant, such as by dialysis against a solution of urea and a combination of reduced and oxidized glutathione, followed by dialysis against a buffered saline solution. In the latter case, the polypeptide can be recovered from the periplasmic space in a soluble and functional form by disrupting the cells (by, for example, sonication or osmotic shock) to release the contents of the periplasmic space and recovering the protein, thereby obviating the need for denaturation and refolding.

Transformed or transfected host cells are cultured according to conventional procedures in a culture medium containing nutrients and other components required for the growth of the chosen host cells. A variety of suitable media, including

defined media and complex media, are known in the art and generally include a carbon source, a nitrogen source, essential amino acids, vitamins and minerals. Media may also contain such components as growth factors or serum, as required. The growth medium will generally select for cells containing the exogenously added DNA by, for example, drug selection or deficiency in an essential nutrient which is complemented by the selectable marker carried on the expression vector or co-transfected into the host cell. Liquid cultures are provided with sufficient aeration by conventional means, such as shaking of small flasks or sparging of fermentors.

Depending upon the intended use, the polypeptides and proteins of the present invention may be purified to ≥80% purity, ≥90% purity, ≥95% purity, or to a pharmaceutically pure state, that is greater than 99.9% pure with respect to contaminating macromolecules, particularly other proteins and nucleic acids, and free of infectious and pyrogenic agents.

Expressed recombinant zalpha48 proteins (including chimeric polypeptides and multimeric proteins) are purified by conventional protein purification methods, typically by a combination of chromatographic techniques. See, in general, Affinity Chromatography: Principles & Methods, Pharmacia LKB Biotechnology, Uppsala, Sweden, 1988; and Scopes, Protein Purification: Principles and Practice, Springer-Verlag, New York, 1994. Proteins comprising a polyhistidine affinity tag (typically about 6 histidine residues) are purified by affinity chromatography on a nickel chelate resin. See, for example, Houchuli et al., *Bio/Technol.* 6: 1321-1325, 1988. Proteins comprising a glu-glu tag can be purified by immunoaffinity chromatography according to conventional procedures. See, for example, Grussenmeyer et al., *ibid.* Maltose binding protein fusions are purified on an amylose column according to methods known in the art.

Zalpha48 polypeptides can also be prepared through chemical synthesis according to methods known in the art, including exclusive solid phase synthesis, partial solid phase methods, fragment condensation or classical solution synthesis. See, for example, Merrifield, *J. Am. Chem. Soc.* 85:2149, 1963; Stewart et al., Solid Phase Peptide Synthesis (2nd edition), Pierce Chemical Co., Rockford, IL, 1984; Bayer and Rapp, *Chem. Pept. Prot.* 3:3, 1986; and Atherton et al., Solid Phase Peptide Synthesis:

A Practical Approach, IRL Press, Oxford, 1989. In vitro synthesis is particularly advantageous for the preparation of smaller polypeptides.

Using methods known in the art, zalpha48 proteins can be prepared as monomers or multimers; glycosylated or non-glycosylated; pegylated or non-pegylated; and may or may not include an initial methionine amino acid residue.

Target cells for use in zalpha48 activity assays include, without limitation, vascular cells (especially endothelial cells and smooth muscle cells), hematopoietic (myeloid and lymphoid) cells, liver cells (including hepatocytes, fenestrated endothelial cells, Kupffer cells, and Ito cells), fibroblasts (including human dermal fibroblasts and lung fibroblasts), fetal lung cells, articular synoviocytes, pericytes, chondrocytes, osteoblasts, and prostate epithelial cells. Endothelial cells and hematopoietic cells are derived from a common ancestral cell, the hemangioblast (Choi et al., *Development* 125:725-732, 1998).

Activity of zalpha48 proteins can be measured *in vitro* using cultured cells or *in vivo* by administering molecules of the claimed invention to an appropriate animal model. Assays measuring cell proliferation or differentiation are well known in the art. For example, assays measuring proliferation include such assays as chemosensitivity to neutral red dye (Cavanaugh et al., *Investigational New Drugs* 8:347-354, 1990), incorporation of radiolabelled nucleotides (as disclosed by, e.g., Raines and Ross, *Methods Enzymol.* 109:749-773, 1985; Wahl et al., *Mol. Cell Biol.* 8:5016-5025, 1988; and Cook et al., *Analytical Biochem.* 179:1-7, 1989), incorporation of 5-bromo-2'-deoxyuridine (BrdU) in the DNA of proliferating cells (Porstmann et al., *J. Immunol. Methods* 82:169-179, 1985), and use of tetrazolium salts (Mosmann, *J. Immunol. Methods* 65:55-63, 1983; Alley et al., *Cancer Res.* 48:589-601, 1988; Marshall et al., *Growth Reg.* 5:69-84, 1995; and Scudiero et al., *Cancer Res.* 48:4827-4833, 1988). Differentiation can be assayed using suitable precursor cells that can be induced to differentiate into a more mature phenotype. Assays measuring differentiation include, for example, measuring cell-surface markers associated with stage-specific expression of a tissue, enzymatic activity, functional activity or morphological changes (Watt, *FASEB*, 5:281-284, 1991; Francis, *Differentiation* 57:63-75, 1994; Raes, Adv. Anim. Cell Biol. Technol. Bioprocesses, 161-171, 1989).

Zalpha48 activity may also be detected using assays designed to measure zalpha48-induced production of one or more additional growth factors or other macromolecules. Such assays include those for determining the presence of hepatocyte growth factor (HGF), epidermal growth factor (EGF), transforming growth factor alpha (TGFα), interleukin-6 (IL-6), VEGF, acidic fibroblast growth factor (aFGF), angiogenin, and other macromolecules produced by the liver. Suitable assays include mitogenesis assays using target cells responsive to the macromolecule of interest, receptor-binding assays, competition binding assays, immunological assays (e.g., ELISA), and other formats known in the art. Metalloprotease secretion is measured from treated primary human dermal fibroblasts, synoviocytes and chondrocytes. The relative levels of collagenase, gelatinase and stromalysin produced in response to culturing in the presence of a zalpha48 protein is measured using zymogram gels (Loita and Stetler-Stevenson, *Cancer Biology* 1:96-106, 1990). Procollagen/collagen synthesis by dermal fibroblasts and chondrocytes in response to a test protein is measured using ^{3}H-proline incorporation into nascent secreted collagen. ^{3}H-labeled collagen is visualized by SDS-PAGE followed by autoradiography (Unemori and Amento, *J. Biol. Chem.* 265: 10681-10685, 1990). Glycosaminoglycan (GAG) secretion from dermal fibroblasts and chondrocytes is measured using a 1,9-dimethylmethylene blue dye binding assay (Farndale et al., *Biochim. Biophys. Acta* 883:173-177, 1986). Collagen and GAG assays are also carried out in the presence of IL-1β or TGF-β to examine the ability of zalpha48 protein to modify the established responses to these cytokines.

Monocyte activation assays are carried out (1) to look for the ability of zalpha48 proteins to further stimulate monocyte activation, and (2) to examine the ability of zalpha48 proteins to modulate attachment-induced or endotoxin-induced monocyte activation (Fuhlbrigge et al., *J. Immunol.* 138: 3799-3802, 1987). IL-1β and TNFα levels produced in response to activation are measured by ELISA (Biosource, Inc. Camarillo, CA). Monocyte/macrophage cells, by virtue of CD14 (LPS receptor), are exquisitely sensitive to endotoxin, and proteins with moderate levels of endotoxin-like activity will activate these cells.

Hematopoietic activity of zalpha48 proteins can be assayed on various hematopoietic cells in culture. Suitable assays include primary bone marrow colony

assays and later stage lineage-restricted colony assays, which are known in the art (e.g., Holly et al., WIPO Publication WO 95/21920). Marrow cells plated on a suitable semi-solid medium (e.g., 50% methylcellulose containing 15% fetal bovine serum, 10% bovine serum albumin, and 0.6% PSN antibiotic mix) are incubated in the presence of test polypeptide, then examined microscopically for colony formation. Known hematopoietic factors are used as controls. Mitogenic activity of zalpha48 polypeptides on hematopoietic cell lines can be measured as disclosed above.

Cell migration is assayed essentially as disclosed by Kähler et al. (*Arteriosclerosis, Thrombosis, and Vascular Biology* 17:932-939, 1997). A protein is considered to be chemotactic if it induces migration of cells from an area of low protein concentration to an area of high protein concentration. A typical assay is performed using modified Boyden chambers with a polystryrene membrane separating the two chambers. Cell migration can also be measured using the matrigel method of Grant et al. ("Angiogenesis as a component of epithelial-mesenchymal interactions" in Goldberg and Rosen, *Epithelial-Mesenchymal Interaction in Cancer*, Birkhäuser Verlag, 1995, 235-248; Baatout, *Anticancer Research* 17:451-456, 1997).

Cell adhesion activity is assayed essentially as disclosed by LaFleur et al. (*J. Biol. Chem.* 272:32798-32803, 1997). Briefly, microtiter plates are coated with the test protein, non-specific sites are blocked with BSA, and cells (such as smooth muscle cells, leukocytes, or endothelial cells) are plated at a density of approximately 10^4 - 10^5 cells/well. The wells are incubated at 37°C (typically for about 60 minutes), then non-adherent cells are removed by gentle washing. Adhered cells are quantitated by conventional methods (e.g., by staining with crystal violet, lysing the cells, and determining the optical density of the lysate). Control wells are coated with a known adhesive protein, such as fibronectin or vitronectin.

The activity of zalpha48 proteins can be measured with a silicon-based biosensor microphysiometer that measures the extracellular acidification rate or proton excretion associated with receptor binding and subsequent physiologic cellular responses. An exemplary such device is the Cytosensor™ Microphysiometer manufactured by Molecular Devices, Sunnyvale, CA. A variety of cellular responses, such as cell proliferation, ion transport, energy production, inflammatory response,

regulatory and receptor activation, and the like, can be measured by this method. See, for example, McConnell et al., *Science* 257:1906-1912, 1992; Pitchford et al., *Meth. Enzymol.* 228:84-108, 1997; Arimilli et al., *J. Immunol. Meth.* 212:49-59, 1998; and Van Liefde et al., *Eur. J. Pharmacol.* 346:87-95, 1998. The microphysiometer can be used for assaying adherent or non-adherent eukaryotic or prokaryotic cells. By measuring extracellular acidification changes in cell media over time, the microphysiometer directly measures cellular responses to various stimuli, including zalpha48 proteins, their agonists, and antagonists. Preferably, the microphysiometer is used to measure responses of a zalpha48-responsive eukaryotic cell, compared to a control eukaryotic cell that does not respond to zalpha48 polypeptide. Zalpha48-responsive eukaryotic cells comprise cells into which a receptor for zalpha48 has been transfected, thereby creating a cell that is responsive to zalpha48, as well as cells naturally responsive to zalpha48. Those skilled in the art will recognize that these methods can also be used to identify agonists and antagonists of zalpha48 proteins.

Expression of zalpha48 polynucleotides in animals provides models for further study of the biological effects of overproduction or inhibition of protein activity *in vivo*. Zalpha48-encoding polynucleotides and antisense polynucleotides can be introduced into test animals, such as mice, using viral vectors or naked DNA, or transgenic animals can be produced.

One *in vivo* approach for assaying proteins of the present invention utilizes viral delivery systems. Exemplary viruses for this purpose include adenovirus, herpesvirus, retroviruses, vaccinia virus, and adeno-associated virus (AAV). Adenovirus, a double-stranded DNA virus, is currently the best studied gene transfer vector for delivery of heterologous nucleic acids. For review, see Becker et al., *Meth. Cell Biol.* 43:161-89, 1994; and Douglas and Curiel, *Science & Medicine* 4:44-53, 1997. The adenovirus system offers several advantages. Adenovirus can (i) accommodate relatively large DNA inserts; (ii) be grown to high-titer; (iii) infect a broad range of mammalian cell types; and (iv) be used with many different promoters including ubiquitous, tissue specific, and regulatable promoters. Because adenoviruses are stable in the bloodstream, they can be administered by intravenous injection.

By deleting portions of the adenovirus genome, larger inserts (up to 7 kb) of heterologous DNA can be accommodated. These inserts can be incorporated into the viral DNA by direct ligation or by homologous recombination with a co-transfected plasmid. In an exemplary system, the essential E1 gene is deleted from the viral vector, and the virus will not replicate unless the E1 gene is provided by the host cell (e.g., the human 293 cell line). When intravenously administered to intact animals, adenovirus primarily targets the liver. If the adenoviral delivery system has an E1 gene deletion, the virus cannot replicate in the host cells. However, the host's tissue (e.g., liver) will express and process (and, if a signal sequence is present, secrete) the heterologous protein. Secreted proteins will enter the circulation in the highly vascularized liver, and effects on the infected animal can be determined.

An alternative method of gene delivery comprises removing cells from the body and introducing a vector into the cells as a naked DNA plasmid. The transformed cells are then re-implanted in the body. Naked DNA vectors are introduced into host cells by methods known in the art, including transfection, electroporation, microinjection, transduction, cell fusion, DEAE dextran, calcium phosphate precipitation, use of a gene gun, or use of a DNA vector transporter. See, Wu et al., *J. Biol. Chem.* 263:14621-14624, 1988; Wu et al., *J. Biol. Chem.* 267:963-967, 1992; and Johnston and Tang, *Meth. Cell Biol.* 43:353-365, 1994.

Transgenic mice, engineered to express a zalpha48 gene, and mice that exhibit a complete absence of zalpha48 gene function, referred to as "knockout mice" (Snouwaert et al., *Science* 257:1083, 1992), can also be generated (Lowell et al., *Nature* 366:740-742, 1993). These mice can be employed to study the zalpha48 gene and the protein encoded thereby in an *in vivo* system. Transgenic mice are particularly useful for investigating the role of zalpha48 proteins in early development in that they allow the identification of developmental abnormalities or blocks resulting from the over- or underexpression of a specific factor. See also, Maisonpierre et al., *Science* 277:55-60, 1997 and Hanahan, *Science* 277:48-50, 1997. Exemplary promoters for transgenic expression include promoters from metallothionein and albumin genes.

Antisense methodology can be used to inhibit zalpha48 gene transcription to examine the effects of such inhibition *in vivo*. Polynucleotides that are

complementary to a segment of a zalpha48-encoding polynucleotide (e.g., a polynucleotide as set forth in SEQ ID NO:1) are designed to bind to zalpha48-encoding mRNA and to inhibit translation of such mRNA. Such antisense oligonucleotides can also be used to inhibit expression of zalpha48 polypeptide-encoding genes in cell culture.

Most four-helix bundle cytokines as well as other proteins produced by activated lymphocytes play an important biological role in cell differentiation, activation, recruitment and homeostasis of cells throughout the body. Zalpha48 and inhibitors of zalpha48 activity are expected to have a variety of therapeutic applications. These therapeutic applications include treatment of diseases which require immune regulation, including autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, myasthenia gravis, systemic lupus erythematosis, and diabetes. Zalpha48 may be important in the regulation of inflammation, and therefore would be useful in treating rheumatoid arthritis, asthma and sepsis. There may be a role of zalpha48 in mediating tumorgenesis, whereby a zalpha48 antagonist would be useful in the treatment of cancer. Zalpha48 may be useful in modulating the immune system, whereby zalpha48 and zalpha48 antagonists may be used for reducing graft rejection, preventing graft-vs-host disease, boosting immunity to infectious diseases, treating immunocompromised patients (e.g., HIV^+ patients), or in improving vaccines.

Zalpha48 polypeptides can be administered alone or in combination with other vasculogenic or angiogenic agents, including VEGF. When using zalpha48 in combination with an additional agent, the two compounds can be administered simultaneously or sequentially as appropriate for the specific condition being treated.

For pharmaceutical use, zalpha48 proteins are formulated for topical or parenteral, particularly intravenous or subcutaneous, delivery according to conventional methods. In general, pharmaceutical formulations will include a zalpha48 polypeptide in combination with a pharmaceutically acceptable vehicle, such as saline, buffered saline, 5% dextrose in water, or the like. Formulations may further include one or more excipients, preservatives, solubilizers, buffering agents, albumin to prevent protein loss on vial surfaces, etc. Methods of formulation are well known in the art and are disclosed, for example, in *Remington: The Science and Practice of Pharmacy*, Gennaro,

ed.. Mack Publishing Co., Easton, PA, 19th ed., 1995. Zalpha48 will preferably be used in a concentration of about 10 to 100 µg/ml of total volume, although concentrations in the range of 1 ng/ml to 1000 µg/ml may be used. For topical application, such as for the promotion of wound healing, the protein will be applied in the range of 0.1-10 $\mu g/cm^2$ of wound area, with the exact dose determined by the clinician according to accepted standards, taking into account the nature and severity of the condition to be treated, patient traits, etc. Determination of dose is within the level of ordinary skill in the art. Dosing is daily or intermittently over the period of treatment. Intravenous administration will be by bolus injection or infusion over a typical period of one to several hours. Sustained release formulations can also be employed. In general, a therapeutically effective amount of zalpha48 is an amount sufficient to produce a clinically significant change in the treated condition, such as a clinically significant change in hematopoietic or immune function, a significant reduction in morbidity, or a significantly increased histological score.

Zalpha48 proteins, agonists, and antagonists are useful for modulating the expansion, proliferation, activation, differentiation, migration, or metabolism of responsive cell types, which include both primary cells and cultured cell lines. Of particular interest in this regard are hematopoietic cells (including stem cells and mature myeloid and lymphoid cells), endothelial cells, smooth muscle cells, fibroblasts, and hepatocytes. Zalpha48 polypeptides are added to tissue culture media for these cell types at a concentration of about 10 pg/ml to about 100 ng/ml. Those skilled in the art will recognize that zalpha48 proteins can be advantageously combined with other growth factors in culture media.

Within the laboratory research field, zalpha48 proteins can also be used as molecular weight standards or as reagents in assays for determining circulating levels of the protein, such as in the diagnosis of disorders characterized by over- or under-production of zalpha48 protein or in the analysis of cell phenotype.

Zalpha48 proteins can also be used to identify inhibitors of their activity. Test compounds are added to the assays disclosed above to identify compounds that inhibit the activity of zalpha48 protein. In addition to those assays disclosed above, samples can be tested for inhibition of zalpha48 activity within a variety of assays

designed to measure receptor binding or the stimulation/inhibition of zalpha48-dependent cellular responses. For example, zalpha48-responsive cell lines can be transfected with a reporter gene construct that is responsive to a zalpha48-stimulated cellular pathway. Reporter gene constructs of this type are known in the art, and will generally comprise a zalpha48-activated serum response element (SRE) operably linked to a gene encoding an assayable protein, such as luciferase. Candidate compounds, solutions, mixtures or extracts are tested for the ability to inhibit the activity of zalpha48 on the target cells as evidenced by a decrease in zalpha48 stimulation of reporter gene expression. Assays of this type will detect compounds that directly block zalpha48 binding to cell-surface receptors, as well as compounds that block processes in the cellular pathway subsequent to receptor-ligand binding. In the alternative, compounds or other samples can be tested for direct blocking of zalpha48 binding to receptor using zalpha48 tagged with a detectable label (e.g., ^{125}I, biotin, horseradish peroxidase, FITC, and the like). Within assays of this type, the ability of a test sample to inhibit the binding of labeled zalpha48 to the receptor is indicative of inhibitory activity, which can be confirmed through secondary assays. Receptors used within binding assays may be cellular receptors or isolated, immobilized receptors.

As used herein, the term "antibodies" includes polyclonal antibodies, monoclonal antibodies, antigen-binding fragments thereof such as $F(ab')_2$ and Fab fragments, single chain antibodies, and the like, including genetically engineered antibodies. Non-human antibodies may be humanized by grafting non-human CDRs onto human framework and constant regions, or by incorporating the entire non-human variable domains (optionally "cloaking" them with a human-like surface by replacement of exposed residues, wherein the result is a "veneered" antibody). In some instances, humanized antibodies may retain non-human residues within the human variable region framework domains to enhance proper binding characteristics. Through humanizing antibodies, biological half-life may be increased, and the potential for adverse immune reactions upon administration to humans is reduced. One skilled in the art can generate humanized antibodies with specific and different constant domains (i.e., different Ig subclasses) to facilitate or inhibit various immune functions associated with particular antibody constant domains. Antibodies are defined to be specifically binding if they

bind to a zalpha48 polypeptide or protein with an affinity at least 10-fold greater than the binding affinity to control (non-zalpha48) polypeptide or protein. The affinity of a monoclonal antibody can be readily determined by one of ordinary skill in the art (see, for example, Scatchard, *Ann. NY Acad. Sci.* 51: 660-672, 1949).

Methods for preparing polyclonal and monoclonal antibodies are well known in the art (see for example, Hurrell, J. G. R., Ed., *Monoclonal Hybridoma Antibodies: Techniques and Applications*, CRC Press, Inc., Boca Raton, FL, 1982). As would be evident to one of ordinary skill in the art, polyclonal antibodies can be generated from a variety of warm-blooded animals such as horses, cows, goats, sheep, dogs, chickens, rabbits, mice, and rats. The immunogenicity of a zalpha48 polypeptide may be increased through the use of an adjuvant such as alum (aluminum hydroxide) or Freund's complete or incomplete adjuvant. Polypeptides useful for immunization also include fusion polypeptides, such as fusions of a zalpha48 polypeptide or a portion thereof with an immunoglobulin polypeptide or with maltose binding protein. The polypeptide immunogen may be a full-length molecule or a portion thereof. If the polypeptide portion is "hapten-like", such portion may be advantageously joined or linked to a macromolecular carrier (such as keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA) or tetanus toxoid) for immunization.

Alternative techniques for generating or selecting antibodies include *in vitro* exposure of lymphocytes to zalpha48 polypeptides, and selection of antibody display libraries in phage or similar vectors (e.g., through the use of immobilized or labeled zalpha48 polypeptide). Human antibodies can be produced in transgenic, non-human animals that have been engineered to contain human immunoglobulin genes as disclosed in WIPO Publication WO 98/24893. It is preferred that the endogenous immunoglobulin genes in these animals be inactivated or eliminated, such as by homologous recombination.

A variety of assays known to those skilled in the art can be utilized to detect antibodies which specifically bind to zalpha48 polypeptides. Exemplary assays are described in detail in *Antibodies: A Laboratory Manual*, Harlow and Lane (Eds.), Cold Spring Harbor Laboratory Press, 1988. Representative examples of such assays include: concurrent immunoelectrophoresis, radio-immunoassays, radio-

immunoprecipitations, enzyme-linked immunosorbent assays (ELISA), dot blot assays, Western blot assays, inhibition or competition assays, and sandwich assays.

Antibodies to zalpha48 may be used for affinity purification of the protein, within diagnostic assays for determining circulating levels of the protein; for detecting or quantitating soluble zalpha48 polypeptide as a marker of underlying pathology or disease; for immunolocalization within whole animals or tissue sections, including immunodiagnostic applications; for immunohistochemistry; and as antagonists to block protein activity *in vitro* and *in vivo*. Antibodies to zalpha48 may also be used for tagging cells that express zalpha48; for affinity purification of zalpha48 polypeptides and proteins; in analytical methods employing FACS; for screening expression libraries; and for generating anti-idiotypic antibodies. For example, antibodies to zalpha48 can be used to tag pancreatic islet cells or cells from adrenal gland, testis, or ovary. Anti-idiotypic antibodies can be linked to other compounds, including therapeutic and diagnostic agents, using known methods, to provide for targetting of those compounds to cells expressing receptors for zalpha48. Antibodies of the present invention may also be directly or indirectly conjugated to drugs, toxins, radionuclides and the like as disclosed below, and these conjugates used for *in vivo* diagnostic or therapeutic applications (e.g., inhibition of cell proliferation) or for *in vitro* diagnosis. See, in general, Ramakrishnan et al., *Cancer Res.* 56:1324-1330, 1996.

Polypeptides and proteins of the present invention can be used to identify and isolate receptors. Zalpha48 receptors may be involved in growth regulation in the liver, blood vessel formation, and other developmental processes. For example, zalpha48 proteins and polypeptides can be immobilized on a column, and membrane preparations run over the column (as generally disclosed in Immobilized Affinity Ligand Techniques, Hermanson et al., eds., Academic Press, San Diego, CA, 1992, pp.195-202). Proteins and polypeptides can also be radiolabeled (*Methods Enzymol.*, vol. 182, "Guide to Protein Purification", M. Deutscher, ed., Academic Press, San Diego, 1990, 721-737) or photoaffinity labeled (Brunner et al., *Ann. Rev. Biochem.* 62:483-514, 1993 and Fedan et al., *Biochem. Pharmacol.* 33:1167-1180, 1984) and used to tag specific cell-surface proteins. In a similar manner, radiolabeled zalpha48 proteins

and polypeptides can be used to clone the cognate receptor in binding assays using cells transfected with an expression cDNA library.

The present invention also provides reagents for use in diagnostic applications. For example, the zalpha48 gene, a probe comprising zalpha48 DNA or RNA, or a subsequence thereof can be used to determine the presence of mutations at or near the zalpha48 locus. Detectable chromosomal aberrations at the zalpha48 gene locus include, but are not limited to, aneuploidy, gene copy number changes, insertions, deletions, restriction site changes, and rearrangements. These aberrations can occur within the coding sequence, within introns, or within flanking sequences, including upstream promoter and regulatory regions, and may be manifested as physical alterations within a coding sequence or changes in gene expression level. Analytical probes will generally be at least 20 nucleotides in length, although somewhat shorter probes (14-17 nucleotides) can be used. PCR primers are at least 5 nucleotides in length, commonly 15 or more nt, and more often 20-30 nt. Short polynucleotides can be used when a small region of the gene is targetted for analysis. For gross analysis of genes, a polynucleotide probe may comprise an entire exon or more. Probes will generally comprise a polynucleotide linked to a signal-generating moiety such as a radionucleotide. In general, these diagnostic methods comprise the steps of (a) obtaining a genetic sample from a patient; (b) incubating the genetic sample with a polynucleotide probe or primer as disclosed above, under conditions wherein the polynucleotide will hybridize to complementary polynucleotide sequence, to produce a first reaction product; and (c) comparing the first reaction product to a control reaction product. A difference between the first reaction product and the control reaction product is indicative of a genetic abnormality in the patient. Genetic samples for use within the present invention include genomic DNA, cDNA, and RNA. The polynucleotide probe or primer can be RNA or DNA, and will comprise a portion of SEQ ID NO:1, the complement of SEQ ID NO:1, or an RNA equivalent thereof. Suitable assay methods in this regard include molecular genetic techniques known to those in the art, such as restriction fragment length polymorphism (RFLP) analysis, short tandem repeat (STR) analysis employing PCR techniques, ligation chain reaction (Barany, *PCR Methods and Applications* 1:5-16, 1991), ribonuclease protection assays,

and other genetic linkage analysis techniques known in the art (Sambrook et al., *ibid.*; Ausubel et. al., *ibid.*; A.J. Marian, *Chest* 108:255-65, 1995). Ribonuclease protection assays (see, e.g., Ausubel et al., *ibid.*, ch. 4) comprise the hybridization of an RNA probe to a patient RNA sample, after which the reaction product (RNA-RNA hybrid) is exposed to RNase. Hybridized regions of the RNA are protected from digestion. Within PCR assays, a patient genetic sample is incubated with a pair of polynucleotide primers, and the region between the primers is amplified and recovered. Changes in size or amount of recovered product are indicative of mutations in the patient. Another PCR-based technique that can be employed is single strand conformational polymorphism (SSCP) analysis (Hayashi, *PCR Methods and Applications* 1:34-38, 1991).

Radiation hybrid mapping is a somatic cell genetic technique developed for constructing high-resolution, contiguous maps of mammalian chromosomes (Cox et al., *Science* 250:245-50, 1990). Partial or full knowledge of a gene's sequence allows one to design PCR primers suitable for use with chromosomal radiation hybrid mapping panels. Radiation hybrid mapping panels that cover the entire human genome are commercially available, such as the Stanford G3 RH Panel and the GeneBridge 4 RH Panel (Research Genetics, Inc., Huntsville, AL). These panels enable rapid, PCR-based chromosomal localizations and ordering of genes, sequence-tagged sites (STSs), and other nonpolymorphic and polymorphic markers within a region of interest, and the establishment of directly proportional physical distances between newly discovered genes of interest and previously mapped markers. The precise knowledge of a gene's position can be useful for a number of purposes, including: 1) determining if a sequence is part of an existing contig and obtaining additional surrounding genetic sequences in various forms, such as YACs, BACs or cDNA clones; 2) providing a possible candidate gene for an inheritable disease which shows linkage to the same chromosomal region; and 3) cross-referencing model organisms, such as mouse, which may aid in determining what function a particular gene might have.

Sequence tagged sites (STSs) can also be used independently for chromosomal localization. An STS is a DNA sequence that is unique in the human genome and can be used as a reference point for a particular chromosome or region of a

chromosome. An STS is defined by a pair of oligonucleotide primers that are used in a polymerase chain reaction to specifically detect this site in the presence of all other genomic sequences. Since STSs are based solely on DNA sequence they can be completely described within an electronic database, for example, Database of Sequence Tagged Sites (dbSTS), GenBank (National Center for Biological Information, National Institutes of Health, Bethesda, MD http://www.ncbi.nlm.nih.gov), and can be searched with a gene sequence of interest for the mapping data contained within these short genomic landmark STS sequences.

Zalpha48 polynucleotides can also be used as probes for analyzing cells and tissues. Zalpha48 mRNA is found at relatively high levels in pancreas (particularly pancreatic islet cells) and adrenal gland, with lower levels of expression in testis and ovary. Thus, zalpha48 mRNA levels provide a useful reference standard for study of these cells and tissues, such as within investigations of physiological states. High level expression in other tissues or cells may be indicative of metabolic abnormalities, including malignant transformation.

The polypeptides, nucleic acid and/or antibodies of the present invention may be used in diagnosis or treatment of disorders associated with cell loss or abnormal cell proliferation (including cancer). Labeled zalpha48 polypeptides may be used for imaging tumors or other sites of abnormal cell proliferation.

Inhibitors of zalpha48 activity (zalpha48 antagonists) include anti-zalpha48 antibodies and soluble zalpha48 receptors, as well as other peptidic and non-peptidic agents (including ribozymes). Such antagonists can be used to block the effects of zalpha48 on cells or tissues. Of particular interest is the use of antagonists of zalpha48 activity in cancer therapy. As early detection methods improve it becomes possible to intervene at earlier times in tumor development, making it feasible to use inhibitors of growth factors to block cell proliferation, angiogenesis, and other events that lead to tumor development and metastasis. Inhibitors are also expected to be useful in adjunct therapy after surgery to prevent the growth of residual cancer cells. Inhibitors can also be used in combination with other cancer therapeutic agents.

In addition to antibodies, zalpha48 inhibitors include small molecule inhibitors and inactive receptor-binding fragments of zalpha48 polypeptides. Inhibitors

are formulated for pharmaceutical use as generally disclosed above, taking into account the precise chemical and physical nature of the inhibitor and the condition to be treated. The relevant determinations are within the level of ordinary skill in the formulation art.

Polynucleotides encoding zalpha48 polypeptides are useful within gene therapy applications where it is desired to increase or inhibit zalpha48 activity. If a mammal has a mutated or absent zalpha48 gene, a zalpha48 gene can be introduced into the cells of the mammal. In one embodiment, a gene encoding a zalpha48 polypeptide is introduced *in vivo* in a viral vector. Such vectors include an attenuated or defective DNA virus, such as, but not limited to, herpes simplex virus (HSV), papillomavirus, Epstein Barr virus (EBV), adenovirus, adeno-associated virus (AAV), and the like. Defective viruses, which entirely or almost entirely lack viral genes, are preferred. A defective virus is not infective after introduction into a cell. Use of defective viral vectors allows for administration to cells in a specific, localized area, without concern that the vector can infect other cells. Examples of particular vectors include, but are not limited to, a defective herpes simplex virus 1 (HSV1) vector (Kaplitt et al., *Molec. Cell. Neurosci.* 2:320-330, 1991); an attenuated adenovirus vector, such as the vector described by Stratford-Perricaudet et al., *J. Clin. Invest.* 90:626-630, 1992; and a defective adeno-associated virus vector (Samulski et al., *J. Virol.* 61:3096-3101, 1987; Samulski et al., *J. Virol.* 63:3822-3888, 1989). Within another embodiment, a zalpha48 gene can be introduced in a retroviral vector as described, for example, by Anderson et al., U.S. Patent No. 5,399,346; Mann et al. *Cell* 33:153, 1983; Temin et al., U.S. Patent No. 4,650,764; Temin et al., U.S. Patent No. 4,980,289; Markowitz et al., *J. Virol.* 62:1120, 1988; Temin et al., U.S. Patent No. 5,124,263; Dougherty et al., WIPO Publication WO 95/07358; and Kuo et al., *Blood* 82:845, 1993. Alternatively, the vector can be introduced by liposome-mediated transfection ("lipofection"). Synthetic cationic lipids can be used to prepare liposomes for *in vivo* transfection of a gene encoding a marker (Felgner et al., *Proc. Natl. Acad. Sci. USA* 84:7413-7417, 1987; Mackey et al., *Proc. Natl. Acad. Sci. USA* 85:8027-8031, 1988). The use of lipofection to introduce exogenous genes into specific organs *in vivo* has certain practical advantages, including molecular targeting of liposomes to specific cells. Directing transfection to particular cell types is particularly advantageous in a tissue with cellular

heterogeneity, such as the pancreas, liver, kidney, and brain. Lipids may be chemically coupled to other molecules for the purpose of targeting. Peptidic and non-peptidic molecules can be coupled to liposomes chemically. Within another embodiment, cells are removed from the body, a vector is introduced into the cells as a naked DNA plasmid, and the transformed cells are re-implanted into the body as disclosed above.

Antisense methodology can be used to inhibit zalpha48 gene transcription in a patient as generally disclosed above.

Zalpha48 polypeptides and anti-zalpha48 antibodies can be directly or indirectly conjugated to drugs, toxins, radionuclides and the like, and these conjugates used for *in vivo* diagnostic or therapeutic applications. For instance, polypeptides or antibodies of the present invention may be used to identify or treat tissues or organs that express a corresponding anti-complementary molecule (receptor or antigen, respectively, for instance). More specifically, zalpha48 polypeptides or anti-zalpha48 antibodies, or bioactive fragments or portions thereof, can be coupled to detectable or cytotoxic molecules and delivered to a mammal having cells, tissues, or organs that express the anti-complementary molecule.

Suitable detectable molecules can be directly or indirectly attached to the polypeptide or antibody, and include radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescent markers, chemiluminescent markers, magnetic particles, and the like. Suitable cytotoxic molecules can be directly or indirectly attached to the polypeptide or antibody, and include bacterial or plant toxins (for instance, diphtheria toxin, *Pseudomonas* exotoxin, ricin, abrin, saporin, and the like), as well as therapeutic radionuclides, such as iodine-131, rhenium-188 or yttrium-90. These can be either directly attached to the polypeptide or antibody, or indirectly attached according to known methods, such as through a chelating moiety. Polypeptides or antibodies can also be conjugated to cytotoxic drugs, such as adriamycin. For indirect attachment of a detectable or cytotoxic molecule, the detectable or cytotoxic molecule may be conjugated with a member of a complementary/anticomplementary pair, where the other member is bound to the polypeptide or antibody portion. For these purposes, biotin/streptavidin is an exemplary complementary/anticomplementary pair.

Polypeptide-toxin fusion proteins or antibody/fragment-toxin fusion proteins may be used for targeted cell or tissue inhibition or ablation, such as in cancer therapy. Of particular interest in this regard are conjugates of a zalpha48 polypeptide and a cytotoxin, which can be used to target the cytotoxin to a tumor or other tissue that is undergoing undesired angiogenesis or neovascularization. Target cells (i.e., those displaying the zalpha48 receptor) bind the zalpha48-toxin conjugate, which is then internalized, killing the cell. The effects of receptor-specific cell killing (target ablation) are revealed by changes in whole animal physiology or through histological examination. Thus, ligand-dependent, receptor-directed cyotoxicity can be used to enhance understanding of the physiological significance of a protein ligand. A preferred such toxin is saporin. Mammalian cells have no receptor for saporin, which is non-toxic when it remains extracellular.

In another embodiment, zalpha48-cytokine fusion proteins or antibody/fragment-cytokine fusion proteins may be used for enhancing *in vitro* cytotoxicity (for instance, that mediated by monoclonal antibodies against tumor targets) and for enhancing *in vivo* killing of target tissues (for example, blood and bone marrow cancers). See, generally, Hornick et al., *Blood* 89:4437-4447, 1997). In general, cytokines are toxic if administered systemically. The described fusion proteins enable targeting of a cytokine to a desired site of action, such as a cell having binding sites for zalpha48, thereby providing an elevated local concentration of cytokine. Suitable cytokines for this purpose include, for example, interleukin-2 and granulocyte-macrophage colony-stimulating factor (GM-CSF). Such fusion proteins may be used to cause cytokine-induced killing of tumors and other tissues undergoing angiogenesis or neovascularization.

The bioactive polypeptide or antibody conjugates described herein can be delivered intravenously, intra-arterially or intraductally, or may be introduced locally at the intended site of action.

In view of the unique combinations of specific physical and chemical properties embodied in zalpha48 polynucleotides and polypeptides, the polynucleotides and polypeptides of the present invention will additionally find use as educational tools as, for example, laboratory kits for courses related to genetics, molecular biology,

protein chemistry, antibody production and analysis, and the like. Such kits will optionally contain one or more of an instruction sheet, a sheet of specifications, one or more standards or controls, and additional reagents. Kits may also contain various combinations of zalpha48 polynucleotides, polypeptides, and antibodies. Due to their unique polynucleotide and polypeptide sequences, molecules of zalpha48 can be used as standards or as "unknowns" for testing purposes. For example, zalpha48 polynucleotides can be used to teach a student how to prepare expression constructs for bacterial, viral, and/or mammalian expression, including fusion constructs, wherein the zalpha48 gene is to be expressed; for determining the restriction endonuclease cleavage sites of the polynucleotides (which cleavage sites will be evident to those skilled in the art from the sequences disclosed herein); for determining mRNA and DNA localization of zalpha48 polynucleotides in tissues (e.g., by Northern blotting, Southern blotting, or polymerase chain reaction); and for identifying related polynucleotides and polypeptides by nucleic acid hybridization. Zalpha48 polypeptides can be used in teaching preparation of antibodies, identification of proteins by Western blotting, protein purification, determination of the mass of expressed zalpha48 polypeptides as a ratio to total protein expressed, identification of peptide cleavage sites, coupling of amino- and carboxyl-terminal tags, amino acid sequence analysis, as well as, but not limited to, monitoring biological activities of both the native and tagged protein (i.e., receptor binding, signal transduction, proliferation, and differentiation) *in vitro* and *in vivo*. Zalpha48 polypeptides can also be used to teach analytical skills such as mass spectrometry; circular dichroism to determine conformation, in particular the locations of the disulfide bonds; x-ray crystallography to determine the three-dimensional structure in atomic detail; and nuclear magnetic resonance spectroscopy to reveal the structure of proteins in solution. For example, a kit containing a zalpha48 polypeptide can be given to the student to analyze in order to develop or test the student's skills. Since the amino acid sequence and other properties of the polypeptide would be known by the instructor, the instructor would then know whether or not the student has correctly analyzed the polypeptide. Antibodies that specifically bind to zalpha48 polypeptides can be used, for example, as teaching aids to instruct students how to prepare affinity chromatography columns to purify zalpha48 polypeptides and how to

perform immunological assays and histological analysis. Anti-zalpha48 antibodies can also be used as tools in the cloning and sequencing of a polynucleotide that encodes an antibody and are thus useful for teaching a student how to design humanized antibodies. Such kits are considered within the scope of the present invention.

The invention is further illustrated by the following non-limiting examples.

Examples

Example 1

RNA extracted from human pancreatic islet cells was reverse transcribed. The first strand cDNA reaction contained 10 µl of human pancreatic islet cell poly d(T)-selected poly $(A)^+$ mRNA (Clontech Laboratories, Inc., Palo Alto, CA) at a concentration of 1.0 mg/ml. and 2 µl of 20 pmole/µl first strand primer SEQ ID NO:6, containing an *Xho* I restriction site. The mixture was heated at 70°C for 2.5 minutes and cooled by chilling on ice. First strand cDNA synthesis was initiated by the addition of 8 µl of first strand buffer (5x SUPERSCRIPT™ buffer; Life Technologies, Gaithersburg, MD), 4 µl of 100 mM dithiothreitol, and 3 µl of a deoxynucleotide triphosphate (dNTP) solution containing 10 mM each of dTTP, dATP, dGTP and 5-methyl-dCTP (Pharmacia LKB Biotechnology, Piscataway, NJ) to the RNA-primer mixture. The reaction mixture was incubated at 40° C for 2 minutes, followed by the addition of 10 µl of 200 U/µl RNase H^- reverse transcriptase (SUPERSCRIPT II®; Life Technologies). The efficiency of the first strand synthesis was analyzed in a parallel reaction by the addition of 10 µCi of ^{32}P-αdCTP to a 5 µl aliquot from one of the reaction mixtures to label the reaction products for analysis. The reaction mixtures were incubated at 40°C for 5 minutes, 45°C for 50 minutes, then 50°C for 10 minutes. Unincorporated ^{32}P-αdCTP in the mixture was removed by chromatography on a 400 pore size gel filtration column (Clontech Laboratories). The unincorporated nucleotides and primers in the unlabeled first strand reaction mixtures were removed by chromatography on 400 pore size gel filtration column. The length of labeled first strand cDNA was determined by agarose gel electrophoresis.

The second strand reaction contained 102 µl of the unlabeled first strand cDNA, 30 µl of 5x polymerase I buffer (125 mM Tris:HCl, pH 7.5, 500 mM KCl, 25 mM $MgCl_2$, 50mM $(NH_4)_2SO_4$), 2.0 µl of 100 mM dithiothreitol, 3.0 µl of a solution containing 10 mM of each deoxynucleotide triphosphate, 7 µl of 5 mM β-NAD, 2.0 µl of 10 U/µl *E. coli* DNA ligase (New England Biolabs; Beverly, MA), 5 µl of 10 U/µl *E. coli* DNA polymerase I (New England Biolabs), and 1.5 µl of 2 U/µl RNase H (Life Technologies). A 10-µl aliquot from one of the second strand synthesis reactions was labeled by the addition of 10 µCi ^{32}P-αdCTP to monitor the efficiency of second strand synthesis. The reactions were incubated at 16°C for two hours, followed by the addition of 1 µl of a 10 mM dNTP solution and 6.0 µl T4 DNA polymerase (10 U/µl, Boehringer Mannheim, Indianapolis, IN), and incubated for an additional 10 minutes at 16°C. Unincorporated ^{32}P-αdCTP was removed by chromatography through a 400 pore size gel filtration column before analysis by agarose gel electrophoresis. The reaction was terminated by the addition of 10.0 µl 0.5 M EDTA and extraction with phenol/chloroform and chloroform followed by ethanol precipitation in the presence of 3.0 M Na acetate and 2 µl of a dye-labeled carrier (Pellet Paint™ Co-Precipitant; Novagen, Madison, WI). The yield of cDNA was estimated to be approximately 2 µg from starting mRNA template of 10 µg.

Eco RI adapters were ligated onto the 5' ends of the cDNA to enable cloning into an expression vector. A 12.5 µl aliquot of cDNA (~2.0 µg) and 3 µl of 69 pmole/µl of *Eco* RI adapter (Pharmacia LKB Biotechnology Inc.) were mixed with 2.5 µl 10x ligase buffer (660 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$), 2.5 µl of 10 mM ATP, 3.5 µl 0.1 M DTT and 1 µl of 15 U/µl T4 DNA ligase (Promega Corp., Madison, WI). The mixture was incubated 1 hour at 5°C, 2 hours at 7.5°C, 2 hours at 10°C, 2 hours at 12.5°C, and 16 hours at 10°C. The reaction was terminated by the addition of 65 µl H_2O and 10 µl 10X H buffer (Boehringer Mannheim, Indianapolis, IN) and incubation at 70°C for 20 minutes.

To facilitate the directional cloning of the cDNA into an expression vector, the cDNA was digested with *Xho* I, resulting in a cDNA having a 5' *Eco* RI cohesive end and a 3' *Xho* I cohesive end. The *Xho* I restriction site at the 3' end of the

cDNA had been previously introduced. Restriction enzyme digestion was carried out in a reaction mixture by the addition of 1.0 µl of 40 U/µl *Xho* I (Boehringer Mannheim, Indianapolis, IN) with incubation at 37°C for 45 minutes. The reaction was terminated by incubation at 70°C for 20 minutes and chromatography through a 400 pore size gel filtration column.

The cDNA was ethanol precipitated, washed with 70% ethanol, air-dried and resuspended in 10.0 µl water, 2 µl of 10X kinase buffer (660 mM Tris-HCl, pH 7.5, 100 mM $MgCl_2$), 0.5 µl 0.1 M DTT, 2 µl 10 mM ATP, 2 µl T4 polynucleotide kinase (10 U/µl, Life Technologies). Following incubation at 37° C for 30 minutes, the cDNA was ethanol precipitated in the presence of 2.5 M ammonium acetate, and electrophoresed on a 0.8% low melt agarose gel. The contaminating adapters and cDNA below 0.6 Kb in length were excised from the gel. The electrodes were reversed, and the cDNA was electrophoresed until concentrated near the lane origin. The area of the gel containing the concentrated cDNA was excised and placed in a microfuge tube, and the approximate volume of the gel slice was determined. An aliquot of water approximately three times the volume of the gel slice (300 µl) and 35 µl 10x β-agarase I buffer (New England Biolabs) was added to the tube, and the agarose was melted by heating to 65°C for 15 minutes. Following equilibration of the sample to 45°C, 3 µl of 1 U/µl β-agarase I (New England Biolabs, Beverly, MA) was added, and the mixture was incubated for 60 minutes at 45°C to digest the agarose. After incubation, 40 µl of 3 M Na acetate was added to the sample, and the mixture was incubated on ice for 15 minutes. The sample was centrifuged at 14,000 x g for 15 minutes at room temperature to remove undigested agarose. The cDNA was ethanol precipitated, washed in 70% ethanol, air-dried and resuspended in 40 µl water.

Following recovery from the gel, the cDNA was cloned into the *Eco* RI and *Xho* I sites of a commercially available phagemid vector (pBluescript® SK(+); Stratagene, La Jolla, CA) and electroporated into *E. coli* host cells (Electromax DH10B™ cells; Life Technologies, Inc.). Bacterial colonies containing known sequences were identified and eliminated from sequence analysis by reiterative cycles of probe hybridization to high-density colony filter arrays (Genome Systems, St. Louis, MI). cDNAs of known genes were pooled in groups of 50 - 100 inserts and were

labeled with ^{32}P-αdCTP using a commercially available labeling kit (Megaprime™ DNA labeling system; Amersham, Arlington Heights, IL). Colonies which did not hybridize to the probe mixture were selected for sequencing. Sequencing was done using an automated DNA sequencer (ABI PRISM™ 377; PE Applied Biosystems, Foster City, CA) using either the T3 or the reverse primer. The resulting data were analyzed, and a database of partial sequences (ESTs) was prepared.

The pancreatic EST database was analyzed using a quasi-threading method to identify assemblies of contiguous sequences that comprised a predicted secretory signal, an alpha-helical region, and an in-frame stop codon upstream of the predicted intiation Met. One such assembly was identified. A corresponding cDNA clone was recovered from the library and sequenced. Analysis indicated that the clone contained a full-length cDNA encoding a secreted polypeptide of 151 amino acid residues (SEQ ID NO:1 and SEQ ID NO:2).

Example 2

An expression plasmid containing all or part of a polynucleotide encoding zalpha48 is constructed via homologous recombination. A fragment of zalpha48 cDNA is isolated by PCR using the polynucleotide sequence of SEQ ID NO: 1 with flanking regions at the 5` and 3` ends corresponding to the vector sequences flanking the zalpha48 insertion point. The primers for PCR each include from 5' to 3' end: 40 bp of flanking sequence from the vector and 17 bp corresponding to the amino and carboxyl termini from the open reading frame of zalpha48.

Ten µl of the 100 µl PCR reaction mixture is run on a 0.8% low-melting-temperature agarose (SeaPlaque GTG®; FMC BioProducts, Rockland, ME) gel with 1 x TBE buffer for analysis. The remaining 90 µl of the reaction misture is precipitated with the addition of 5 µl 1 M NaCl and 250 µl of absolute ethanol. The plasmid pZMP6, which has been cut with SmaI, is used for recombination with the PCR fragment. Plamid pZMP6 is a mammalian expression vector containing an expression cassette having the cytomegalovirus immediate early promoter, multiple restriction sites for insertion of coding sequences, a stop codon, and a human growth hormone terminator; an *E. coli* origin of replication; a mammalian selectable marker expression

unit comprising an SV40 promoter. enhancer and origin of replication, a DHFR gene, and the SV40 terminator; and URA3 and CEN-ARS sequences required for selection and replication in *S. cerevisiae*. It was constructed from pZP9 (deposited at the American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209, under Accession No. 98668) with the yeast genetic elements taken from pRS316 (deposited at the American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209, under Accession No. 77145), an internal ribosome entry site (IRES) element from poliovirus, and the extracellular domain of CD8 truncated at the C-terminal end of the transmembrane domain.

One hundred microliters of competent yeast (*S. cerevisiae*) cells are independently combined with 10 µl of the various DNA mixtures from above and transferred to a 0.2-cm electroporation cuvette. The yeast/DNA mixtures are electropulsed using power supply (BioRad Laboratories, Hercules, CA) settings of 0.75 kV (5 kV/cm), ∞ ohms, 25 µF. To each cuvette is added 600 µl of 1.2 M sorbitol, and the yeast is plated in two 300-µl aliquots onto two URA-D plates and incubated at 30°C. After about 48 hours, the Ura^+ yeast transformants from a single plate are resuspended in 1 ml H_2O and spun briefly to pellet the yeast cells. The cell pellet is resuspended in 1 ml of lysis buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris, pH 8.0, 1 mM EDTA). Five hundred microliters of the lysis mixture is added to an Eppendorf tube containing 300 µl acid-washed glass beads and 200 µl phenol-chloroform, vortexed for 1 minute intervals two or three times, and spun for 5 minutes in an Eppendorf centrifuge at maximum speed. Three hundred microliters of the aqueous phase is transferred to a fresh tube, and the DNA is precipitated with 600 µl ethanol (EtOH), followed by centrifugation for 10 minutes at 4°C. The DNA pellet is resuspended in 10 µl H_2O.

Transformation of electrocompetent *E. coli* host cells (Electromax DH10B™ cells; obtained from Life Technologies, Inc., Gaithersburg, MD) is done with 0.5-2 ml yeast DNA prep and 40 µl of cells. The cells are electropulsed at 1.7 kV, 25 µF, and 400 ohms. Following electroporation, 1 ml SOC (2% Bacto™ Tryptone (Difco, Detroit, MI), 0.5% yeast extract (Difco), 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10

mM $MgSO_4$, 20 mM glucose) is plated in 250-µl aliquots on four LB AMP plates (LB broth (Lennox), 1.8% Bacto™ Agar (Difco), 100 mg/L Ampicillin).

Individual clones harboring the correct expression construct for zalpha48 are identified by restriction digest to verify the presence of the zalpha48 insert and to confirm that the various DNA sequences have been joined correctly to one another. The inserts of positive clones are subjected to sequence analysis. Larger scale plasmid DNA is isolated using a commercially available kit (QIAGEN Plasmid Maxi Kit, Qiagen, Valencia, CA) according to manufacturer's instructions. The correct construct is designated pZMP6/zalpha48.

Example 3

CHO DG44 cells (Chasin et al., *Som. Cell. Molec. Genet.* 12:555-666, 1986) are plated in 10-cm tissue culture dishes and allowed to grow to approximately 50% to 70% confluency overnight at 37°C, 5% CO_2, in Ham's F12/FBS media (Ham's F12 medium (Life Technologies), 5% fetal bovine serum (Hyclone, Logan, UT), 1% L-glutamine (JRH Biosciences, Lenexa, KS), 1% sodium pyruvate (Life Technologies)). The cells are then transfected with the plasmid zalpha48/pZMP6 by liposome-mediated transfection using a 3:1 (w/w) liposome formulation of the polycationic lipid 2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propaniminium-trifluoroacetate and the neutral lipid dioleoyl phosphatidylethanolamine in membrane-filetered water (Lipofectamine™ Reagent, Life Technologies), in serum free (SF) media formulation (Ham's F12, 10 mg/ml transferrin, 5 mg/ml insulin, 2 mg/ml fetuin, 1% L-glutamine and 1% sodium pyruvate). Zalpha48/pZMP6 is diluted into 15-ml tubes to a total final volume of 640 µl with SF media. 35 µl of Lipofectamine™ is mixed with 605 µl of SF medium. The resulting mixture is added to the DNA mixture and allowed to incubate approximately 30 minutes at room temperature. Five ml of SF media is added to the DNA:Lipofectamine™ mixture. The cells are rinsed once with 5 ml of SF media, aspirated, and the DNA:Lipofectamine™ mixture is added. The cells are incubated at 37°C for five hours, then 6.4 ml of Ham's F12/10% FBS, 1% PSN media is added to each plate. The plates are incubated at 37°C overnight, and the DNA:Lipofectamine™ mixture is replaced with fresh 5% FBS/Ham's media the next

day. On day 3 post-transfection, the cells are split into T-175 flasks in growth medium. On day 7 postransfection, the cells are stained with FITC-anti-CD8 monoclonal antibody (Pharmingen, San Diego, CA) followed by anti-FITC-conjugated magnetic beads (Miltenyi Biotec). The CD8-positive cells are separated using commercially available columns (mini-MACS columns; Miltenyi Biotec) according to the manufacturer's directions and put into DMEM/Ham's F12/5% FBS without nucleosides but with 50 nM methotrexate (selection medium).

Cells are plated for subcloning at a density of 0.5, 1 and 5 cells per well in 96-well dishes in selection medium and allowed to grow out for approximately two weeks. The wells are checked for evaporation of medium and brought back to 200 µl per well as necessary during this process. When a large percentage of the colonies in the plate are near confluency, 100 µl of medium is collected from each well for analysis by dot blot. and the cells are fed with fresh selection medium. The supernatant is applied to a nitrocellulose filter in a dot blot apparatus, and the filter is treated at 100°C in a vacuum oven to denature the protein. The filter is incubated in 625 mM Tris-glycine, pH 9.1, 5mM β-mercaptoethanol, at 65°C, 10 minutes, then in 2.5% non-fat dry milk Western A Buffer (0.25% gelatin, 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.05% Igepal CA-630) overnight at 4°C on a rotating shaker. The filter is incubated with the antibody-HRP conjugate in 2.5% non-fat dry milk Western A buffer for 1 hour at room temperature on a rotating shaker. The filter is then washed three times at room temperature in PBS plus 0.01% Tween 20, 15 minutes per wash. The filter is developed with chemiluminescence reagents (ECL™ direct labelling kit; Amersham Corp., Arlington Heights, IL) according to the manufacturer's directions and exposed to film (Hyperfilm ECL, Amersham Corp.) for approximately 5 minutes. Positive clones are trypsinized from the 96-well dish and transferred to 6-well dishes in selection medium for scaleup and analysis by Western blot.

Example 4

Full-length zalpha48 protein is produced in BHK cells transfected with pZMP6/zalpha48 (Example 2). BHK 570 cells (ATCC CRL-10314) are plated in 10-cm tissue culture dishes and allowed to grow to approximately 50 to 70% confluence

overnight at 37°C, 5% CO_2, in DMEM/FBS media (DMEM, Gibco/BRL High Glucose; Life Technologies), 5% fetal bovine serum (Hyclone, Logan, UT), 1 mM L-glutamine (JRH Biosciences, Lenexa, KS), 1 mM sodium pyruvate (Life Technologies). The cells are then transfected with pZMP6/zalpha48 by liposome-mediated transfection (using Lipofectamine™; Life Technologies), in serum free (SF) media (DMEM supplemented with 10 mg/ml transferrin, 5 mg/ml insulin, 2 mg/ml fetuin, 1% L-glutamine and 1% sodium pyruvate). The plasmid is diluted into 15-ml tubes to a total final volume of 640 µl with SF media. 35 µl of the lipid mixture is mixed with 605 µl of SF medium, and the resulting mixture is allowed to incubate approximately 30 minutes at room temperature. Five milliliters of SF media is then added to the DNA:lipid mixture. The cells are rinsed once with 5 ml of SF media, aspirated, and the DNA:lipid mixture is added. The cells are incubated at 37°C for five hours, then 6.4 ml of DMEM/10% FBS, 1% PSN media is added to each plate. The plates are incubated at 37°C overnight, and the DNA:lipid mixture is replaced with fresh 5% FBS/DMEM media the next day. On day 5 post-transfection, the cells are split into T-162 flasks in selection medium (DMEM + 5% FBS, 1% L-Gln, 1% NaPyr, 1 µM methotrexate). Approximately 10 days post-transfection, two 150-mm culture dishes of methotrexate-resistant colonies from each transfection are trypsinized, and the cells are pooled and plated into a T-162 flask and transferred to large-scale culture.

Example 5

For construction of adenovirus vectors, the protein coding region of human zalpha48 is amplified by PCR using primers that add PmeI and AscI restriction sites at the 5' and 3' termini respectively. Amplification is performed with a full-length zalpha48 cDNA template in a PCR reaction as follows: one cycle at 95°C for 5 minutes; followed by 15 cycles at 95°C for 1 min., 61°C for 1 min., and 72°C for 1.5 min.; followed by 72°C for 7 min.; followed by a 4°C soak. The PCR reaction product is loaded onto a 1.2% low-melting-temperature agarose gel in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA). The zalpha48 PCR product is excised from the gel and purified using a commercially available kit comprising a silica gel mambrane spin column (QIAquick™ PCR Purification Kit and gel cleanup kit; Qiagen, Inc.) as per kit

instructions. The PCR product is then digested with PmeI and AscI, phenol/chloroform extracted, EtOH precipitated, and rehydrated in 20 ml TE (Tris/EDTA pH 8). The zalpha48 fragment is then ligated into the PmeI-AscI sites of the transgenic vector pTG12-8 and transformed into *E. coli* DH10B™ competent cells by electroporation. Vector pTG12-8 was derived from p2999B4 (Palmiter et al., *Mol. Cell Biol.* 13:5266-5275, 1993) by insertion of a rat insulin II intron (ca. 200 bp) and polylinker (Fse I/Pme I/Asc I) into the Nru I site. The vector comprises a mouse metallothionein (MT-1) promoter (ca. 750 bp) and human growth hormone (hGH) untranslated region and polyadenylation signal (ca. 650 bp) flanked by 10 kb of MT-1 5' flanking sequence and 7 kb of MT-1 3' flanking sequence. The cDNA is inserted between the insulin II and hGH sequences. Clones containing zalpha48 are identified by plasmid DNA miniprep followed by digestion with PmeI and AscI. A positive clone is sequenced to insure that there were no deletions or other anomalies in the construct.

DNA is prepared using a commercially available kit (Maxi Kit, Qiagen, Inc.), and the zalpha48 cDNA is released from the pTG12-8 vector using PmeI and AscI enzymes. The cDNA is isolated on a 1% low melting temperature agarose gel and excised from the gel. The gel slice is melted at 70°C, and the DNA is extracted twice with an equal volume of Tris-buffered phenol, precipitated with EtOH, and resuspended in 10 µl H_2O.

The zalpha48 cDNA is cloned into the EcoRV-AscI sites of a modified pAdTrack-CMV (He, T-C. et al., *Proc. Natl. Acad. Sci. USA* 95:2509-2514, 1998). This construct contains the green fluorescent protein (GFP) marker gene. The CMV promoter driving GFP expression is replaced with the SV40 promoter, and the SV40 polyadenylation signal is replaced with the human growth hormone polyadenylation signal. In addition, the native polylinker is replaced with FseI, EcoRV, and AscI sites. This modified form of pAdTrack-CMV is named pZyTrack. Ligation is performed using a commercially available DNA ligation and screening kit (Fast-Link™ kit; Epicentre Technologies, Madison, WI). Clones containing zalpha48 are identified by digestion of mini prep DNA with FseI and AscI.

In order to linearize the plasmid, approximately 5 µg of the resulting pZyTrack zalpha48 plasmid is digested with PmeI. Approximately 1 µg of the

linearized plasmid is cotransformed with 200 ng of supercoiled pAdEasy (He et al., *ibid.*) into *E. coli* BJ5183 cells (He et al., *ibid.*). The co-transformation is done using a Bio-Rad Gene Pulser at 2.5 kV, 200 ohms and 25 μFa. The entire co-transformation mixture is plated on 4 LB plates containing 25 μg/ml kanamycin. The smallest colonies are picked and expanded in LB/kanamycin, and recombinant adenovirus DNA is identified by standard DNA miniprep procedures. The recombinant adenovirus miniprep DNA is transformed into *E. coli* DH10B™ competent cells, and DNA is prepared using a Maxi Kit (Qiagen, Inc.) aaccording to kit instructions.

Approximately 5 μg of recombinant adenoviral DNA is digested with PacI enzyme (New England Biolabs) for 3 hours at 37°C in a reaction volume of 100 μl containing 20-30U of PacI. The digested DNA is extracted twice with an equal volume of phenol/chloroform and precipitated with ethanol. The DNA pellet is resuspended in 10μl distilled water. A T25 flask of QBI-293A cells (Quantum Biotechnologies, Inc. Montreal, Qc. Canada), inoculated the day before and grown to 60-70% confluence, is transfected with the PacI digested DNA. The PacI-digested DNA is diluted up to a total volume of 50 μl with sterile HBS (150mM NaCl, 20mM HEPES). In a separate tube, 20 μl of 1mg/ml N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethyl-ammonium salts (DOTAP) (Boehringer Mannheim, Indianapolis, IN) is diluted to a total volume of 100 μl with HBS. The DNA is added to the DOTAP, mixed gently by pipeting up and down, and left at room temperature for 15 minutes. The media is removed from the 293A cells and washed with 5 ml serum-free minimum essential medium (MEM) alpha containing 1mM sodium pyruvate, 0.1 mM MEM non-essential amino acids, and 25mM HEPES buffer (reagents obtained from Life Technologies, Gaithersburg, MD). 5 ml of serum-free MEM is added to the 293A cells and held at 37°C. The DNA/lipid mixture is added drop-wise to the T25 flask of 293A cells, mixed gently, and incubated at 37°C for 4 hours. After 4 hours the media containing the DNA/lipid mixture is aspirated off and replaced with 5 ml complete MEM containing 5% fetal bovine serum. The transfected cells are monitored for GFP expression and formation of foci (viral plaques).

Seven days after transfection of 293A cells with the recombinant adenoviral DNA, the cells express the GFP protein and start to form foci (viral "plaques"). The crude viral lysate is collected using a cell scraper to collect all of the

293A cells. The lysate is transferred to a 50-ml conical tube. To release most of the virus particles from the cells, three freeze/thaw cycles are done in a dry ice/ethanol bath and a 37° waterbath.

The crude lysate is amplified (Primary (1°) amplification) to obtain a working "stock" of zalpha48 rAdV lysate. Ten 10cm plates of nearly confluent (80-90%) 293A cells are set up 20 hours previously, 200 ml of crude rAdV lysate is added to each 10-cm plate, and the cells are monitored for 48 to 72 hours for CPE (cytopathic effect) under the white light microscope and expression of GFP under the fluorescent microscope. When all of the 293A cells show CPE, this 1° stock lysate is collected and freeze/thaw cycles performed as described above.

A secondary (2°) amplification of zalpha48 rAdV is then performed. Twenty 15-cm tissue culture dishes of 293A cells are prepared so that the cells are 80-90% confluent. All but 20 ml of 5% MEM media is removed, and each dish is inoculated with 300-500 ml of the 1° amplified rAdv lysate. After 48 hours the 293A cells are lysed from virus production, the lysate is collected into 250-ml polypropylene centrifuge bottles, and the rAdV is purified.

NP-40 detergent is added to a final concentration of 0.5% to the bottles of crude lysate in order to lyse all cells. Bottles are placed on a rotating platform for 10 minutes agitating as fast as possible without the bottles falling over. The debris is pelleted by centrifugation at 20,000 X G for 15 minutes. The supernatant is transferred to 250-ml polycarbonate centrifuge bottles, and 0.5 volume of 20% PEG8000/2.5 M NaCl solution is added. The bottles are shaken overnight on ice. The bottles are centrifuged at 20,000 X G for 15 minutes, and the supernatant is discarded into a bleach solution. Using a sterile cell scraper, the white, virus/PEG precipitate from 2 bottles is resuspended in 2.5 ml PBS. The resulting virus solution is placed in 2-ml microcentrifuge tubes and centrifuged at 14,000 X G in the microcentrifuge for 10 minutes to remove any additional cell debris. The supernatant from the 2-ml microcentrifuge tubes is transferred into a 15-ml polypropylene snapcap tube and adjusted to a density of 1.34 g/ml with CsCl. The solution is transferred to 3.2-ml, polycarbonate, thick-walled centrifuge tubes and spun at 348,000 X G for 3-4 hours at

25°C. The virus forms a white band. Using wide-bore pipette tips, the virus band is collected.

A commercially available ion-exchange columns (e.g., PD-10 columns prepacked with Sephadex® G-25M; Pharmacia Biotech, Piscataway, NJ) is used to desalt the virus preparation. The column is equilibrated with 20 ml of PBS. The virus is loaded and allowed to run into the column. 5 ml of PBS is added to the column, and fractions of 8-10 drops are collected. The optical densities of 1:50 dilutions of each fraction are determined at 260 nm on a spectrophotometer. Peak fractions are pooled, and the optical density (OD) of a 1:25 dilution is determined. OD is converted to virus concentration using the formula: (OD at 260nm)(25)(1.1×10^{12}) = virions/ml.

To store the virus, glycerol is added to the purified virus to a final concentration of 15%, mixed gently but effectively, and stored in aliquots at -80°C.

A protocol developed by Quantum Biotechnologies, Inc. (Montreal, Canada) is followed to measure recombinant virus infectivity. Briefly, two 96-well tissue culture plates are seeded with 1×10^4 293A cells per well in MEM containing 2% fetal bovine serum for each recombinant virus to be assayed. After 24 hours 10-fold dilutions of each virus from $1X10^{-2}$ to $1X10^{-14}$ are made in MEM containing 2% fetal bovine serum. 100 µl of each dilution is placed in each of 20 wells. After 5 days at 37°C, wells are read either positive or negative for CPE, and a value for "Plaque Forming Units/ml" (PFU) is calculated.

An adenovirus vector comprising the human zalpha48 coding sequence was constructed essentially as disclosed above and designated AdZyAlpha48.

Example 6

Analysis of tissue distribution was performed by the Northern blotting technique using commercially available blots of human RNA (Human Multiple Tissue Northern Blots; Clontech Laboratories, Inc., Palo Alto, CA). A probe was obtained by restriction digest of a human zalpha48 clone with EcoRI and XhoI, resulting in a cDNA fragment of 470 bp. The fragment was gel-purified, labeled with ^{32}P, and purified using commercially available reagents and standard procedures. Hybridization was carried out using standard procedures and reagents, with washing in 0.1 x SSC and 0.1% SDS at

55°C. The blots were exposed to film for three days at -80°C. One major transcript size was observed on the blots at ~1.4 – 1.5 kb. The signal was strongest in pancreas and adrenal gland, with a weaker signal in testis and ovary.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

CLAIMS

What is claimed is:

1. An isolated polypeptide comprising at least nine contiguous amino acid residues of SEQ ID NO:2.

2. The isolated polypeptide of claim 1 having from 15 to 1500 amino acid residues.

3. The isolated polypeptide of claim 2, wherein said at least nine contiguous amino acid residues of SEQ ID NO:2 are operably linked via a peptide bond or polypeptide linker to a second polypeptide selected from the group consisting of maltose binding protein, an immunoglobulin constant region, a polyhistidine tag, and a peptide as shown in SEQ ID NO:5.

4. The isolated polypeptide of claim 1 comprising at least 30 contiguous residues of SEQ ID NO:2.

5. The isolated polypeptide of claim 1 comprising:
residues 34-48 of SEQ ID NO:2
residues 49-69 of SEQ ID NO:2;
residues 70-84 of SEQ ID NO:2;
residues 87-101 of SEQ ID NO:2;
residues 102-126 of SEQ ID NO:2;
residues 127-141 of SEQ ID NO:2;
residues 20-53 of SEQ ID NO:2; or
residues 49-57 of SEQ ID NO:2.

6. The isolated polypeptide of claim 1 comprising residues 34-147 of SEQ ID NO:3, residues 34-151 of SEQ ID NO:3, or residues 21-151 of SEQ ID NO:3.

7. The isolated polypeptide of claim 1 comprising residues 34-147 of SEQ ID NO:2, residues 34-151 of SEQ ID NO:2, or residues 21-151 of SEQ ID NO:2.

8. An expression vector comprising the following operably linked elements:

a transcription promoter;

a DNA segment encoding a polypeptide comprising a sequence of amino acid residues selected from the group consisting of:

residues 34-48 of SEQ ID NO:2

residues 49-69 of SEQ ID NO:2;

residues 70-84 of SEQ ID NO:2;

residues 87-101 of SEQ ID NO:2;

residues 102-126 of SEQ ID NO:2;

residues 127-141 of SEQ ID NO:2;

residues 20-53 of SEQ ID NO:2; and

residues 49-57 of SEQ ID NO:2; and

a transcription terminator.

9. The expression vector of claim 8 wherein the DNA segment comprises nucleotides 61 to 453 of SEQ ID NO:4.

10. The expression vector of claim 8 wherein the polypeptide comprises residues 34-147 of SEQ ID NO:3, residues 34-151 of SEQ ID NO:3, or residues 21-151 of SEQ ID NO:3.

11. The expression vector of claim 8 wherein the polypeptide comprises residues 34-147 of SEQ ID NO:2, residues 34-151 of SEQ ID NO:2, or residues 21-151 of SEQ ID NO:2.

12. The expression vector of claim 8 further comprising a secretory signal sequence operably linked to the DNA segment.

13. The expression vector of claim 12, wherein the secretory signal sequence encodes residues 1-20 of SEQ ID NO:2.

14. A cultured cell into which has been introduced the expression vector of any of claims 8-13, wherein said cell expresses said DNA segment.

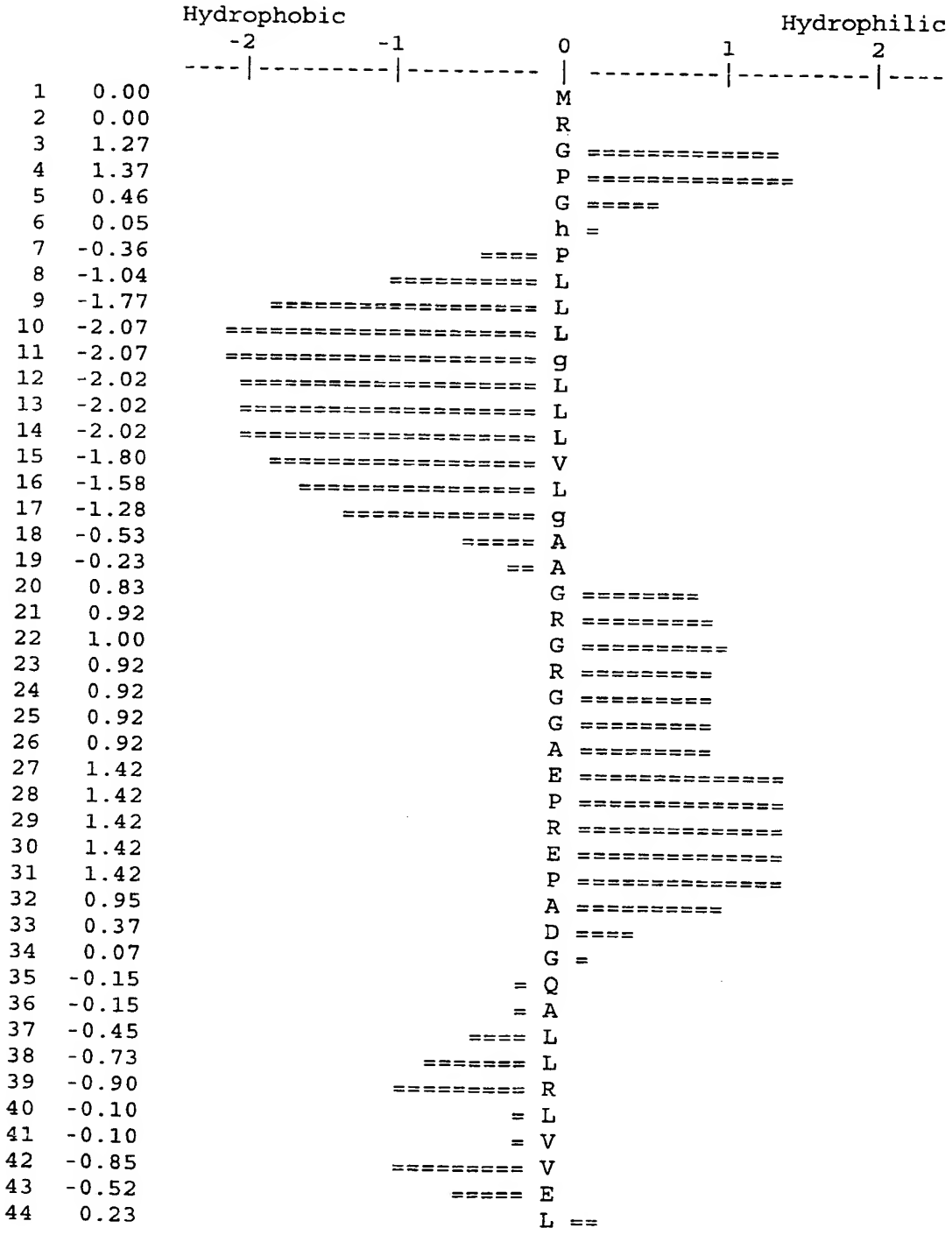
15. A method of making a polypeptide comprising:

culturing a cell into which has been introduced the expression vector of any of claims 8-13 under conditions whereby the DNA segment is expressed and the polypeptide is produced; and

recovering the produced polypeptide.

16. A polypeptide produced by the method of claim 15.

17. An antibody that specifically binds to the polypeptide of claim 4.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43 -0.52 E
44 0.23 L

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78	0.25	P ===
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82	1.28	Q ============
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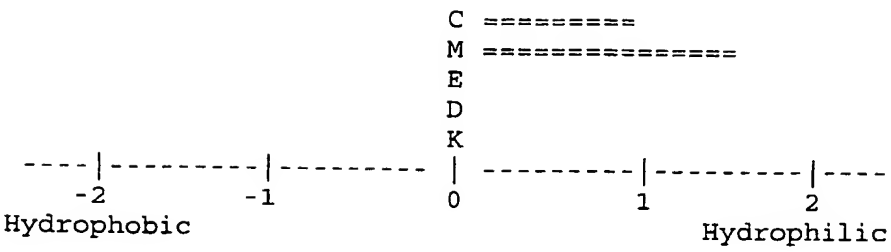

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